AD_____

Award Number: W81XWH-05-1-0126

TITLE: Pim Protein Kinase-Levels Correlate with Prostate Tumor Growth and Chemo-Resistance - Potential Mechanism of PIM Action

PRINCIPAL INVESTIGATOR: Andrew S. Kraft, M.D.

CONTRACTING ORGANIZATION: Medical University of South Carolina
                                              Charleston, South Carolina   29425

REPORT DATE: December 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                         Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                                         Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
01-12-2006	Annual	30 Nov 2005 – 29 Nov 2006

4. TITLE AND SUBTITLE

Pim Protein Kinase-Levels Correlate with Prostate Tumor Growth and Chemo-Resistance - Potential Mechanism of PIM Action

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0126

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Andrew S. Kraft, M.D.

E-Mail: kraft@musc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Medical University of South Carolina
Charleston, South Carolina   29425

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Pim is a protein kinase that has been implicated to play a role in prostate cancer. Kaplan-Meier analysis demonstrated that those patients with high Pim-1 expression are at a significantly greater risk for developing metastatic cancer. In transgenic animal models, the levels of Pim-1 protein kinase are elevated in prostate tumors caused by over expression of the c-myc ongoene, a gene overexpressed in human prostate cancer. In this proposal, we will examine whether Pim mimics Akt and TOR or modulates additional biochemical pathways and use knockout mice to dissect how myc and Pim collaborate to induce transformation and growth or prostate cancers. Pim inhibitors alone or in combination with TOR inhibitors will make a powerful chemotherapeutic strategy.

15. SUBJECT TERMS
Pim protein kinase, TOR, rapamycin, c-Myc, PTEN

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** (include area code)
U	U	U	UU	39	

Table of Contents

Introduction

The **purpose** of this research is to (1) determine using human prostate cancer samples whether Pim is over expressed in tumors with normal PTEN levels, (2) decipher the biochemical mechanism of action by which Pim regulates growth of prostate cancer cells, and (3) to use transgenic mice to understand whether Pim over expression is necessary for c-Myc induced growth of prostate cancer cells. The **scope** of this research involves studying normal prostate epithelial cells in tissue culture, malignant human prostate cancer cell lines, and nude mice with subcutaneous tumors, and transgeneic mice specifically expressing these genes in mice. The research sponsored by this proposal has led to the publication of an article in <u>Molecular Cancer Research</u>, and a paper accepted in <u>Oncogene </u>both dealing with the mechanism of action of the Pim-1 protein kinase.

Body

Task 1 - The goal of this task was to determine how Pim-1 levels are regulated in prostate cancer tissues.   In the first year of this proposal we have seen that Pim appears to interact with PP2A. This is an important interaction because it suggests that an enzyme, PP2A, that controls both the c-Myc and TOR pathways may also be regulating Pim protein kinase.
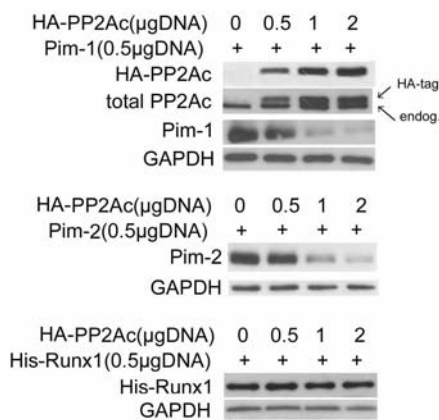
Figure 1 Pim-1 protein levels are negativ[...] PP2Ac. (a) Increased cellular PP2A activity decreases Pim-1 and Pim-2 protein levels. HEK-293T cells were contransfected with 0.5 µg pcDNA3/ Pim-1 or Pim-2, Runx1, and increasing amounts of pD30-PP2A-HA-C, 0.5 µg, 1 µg to 2 µg, as indicated. Control empty pcDNA3 vector was added to ensure that the total amount of plasmid DNA per transfection was identical. Whole-cell lysates were collected at 36 h post-transfection. Western blotting was carried out using anti-HA (for exogenous PP2Ac), anti-PP2Ac (for total PP2Ac), anti-Pim-1, anti-Pim2, and anti-His for the detection of the Runx1 control, and anti-GAPDH another control.

To evaluate the effect of PP2A on the levels of Pim we transfected 293T cells and prostate cells (data not shown) with Pim proteins and also with HA-PP2Ac.
Now we demonstrate that PP2A levels appear to control the levels of Pim-1. This result can be seen in Figure 1 where increases in PP2A led to decreases in Pim-1 protein levels. Thus, increases in PP2A activity enhance both the degradation of Pim-1 and the c-Myc protein. This could suggest that in tumors the levels of PP2A must be regulated to modulate prostate cancer tumor growth.

Figure 2 PP2A dephosphorylates Pim-1 *in vitro*, and decreases Pim-1 kinase activity. *In vitro* dephosphorylation assay. HEK 293T cells were transfected with 2 µg pcDNA3/Flag-Pim-1. After 36 h transfection, the Flag-Pim-1 proteins were labeled by incubating the cells in media containing [^{32}P] orthophosphate for 4 h followed by the immunoprecipitation of Flag-Pim-1 proteins.
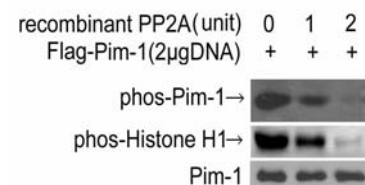
This decrease in Pim-1 protein levels is associated with a decrease in the ability of Pim-1 to phosphorylate its substrates (Figure 2). To test this possibility the [^{32}P] orthophosphate labeled-Flag-tagged Pim immunoprecipitates were treated with or without recombinant PP2A (A/C dimer) in an *in vitro* phosphatase reactions. 40% of the reaction products were analyzed by autoradiography (upper row), 10% were used in a Western blotting for measuring the total amount of Pim-1 in each group by using anti-Pim-1 antibody (bottom row), and 50% of the immunoprecipitate was washed and then subjected to a kinase assay with histone H1 as a substrate (middle row). As can be seen increases in PP2A lead to decreases in Pim activity.

Task 2- The goal of this next task was to understand how PP2A is interacting physically with the Pim-1 protein. The PP2A protein is composed of an A, B, and C subunit. The A subunit works as a scaffold, while the B subunit targets this phosphatase to specific substrates, and the C subunit is the catalytic moiety. To discover how Pim is regulated by PP2A we have incubated Pim with varied PP2A beta subunits. We find that Pim-1 binds tightly to the B56beta subunit as shown in this coimmunoprecipitation experiment illustrated below in Figure 3.
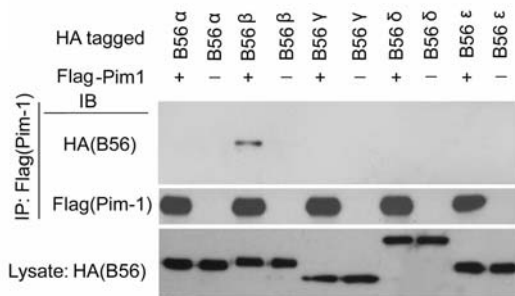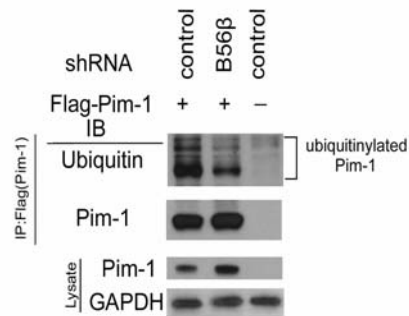

Figure 3 Pim-1 specifically associates with B56β *in vivo*. (a) Pim-1 coimmunoprecipitates with the B56β subunit. Pim-1 was contransfected with 0.5 μg HA tagged B56α, -β, -γ, -δ, -ε subunits into 293T cells. Whole-cell lysates were collected at 48 h posttransfection and immunoprecipitated with anti-HA beads. 5% of the lysates were saved for input detection and 50% of the immunoprecipitation samples were analyzed by Western blotting with anti-HA to recognize the B56 subunits, and anti-Flag to measure Pim-1.

To examine what portion of the Pim-1 molecule bind to PP2A we generated cut backs in Pim-1 protein cDNA and expressed these in cells along with PP2A. We find by this technique that the sequence between 140 and 177 is essential for the interaction of these two proteins. This result is illustrated in Figure 4. These studies demonstrate the close physical interaction between PP2A beta subunit and Pim-1.

Figure 4 Identification of Pim-1 domain responsible for banding to B56β. (a) Structural domains of Pim-1 and Pim-1 deletion mutants use in these experiments are represented as black bars. (b) B56β binds the hinge region of Pim-1. HEK-293T cells were cotransfected with the HA-B56β (full length) along with the empty cDNA (mock) or the indicated Flag-tagged Pim-1 deletion mutants. The Flag-Pim-1 proteins were immunoprecipitated with anti-Flag beads and immunoblotted with anti-HA antibody to detect B56β or anti-Flag to measure Pim-1.
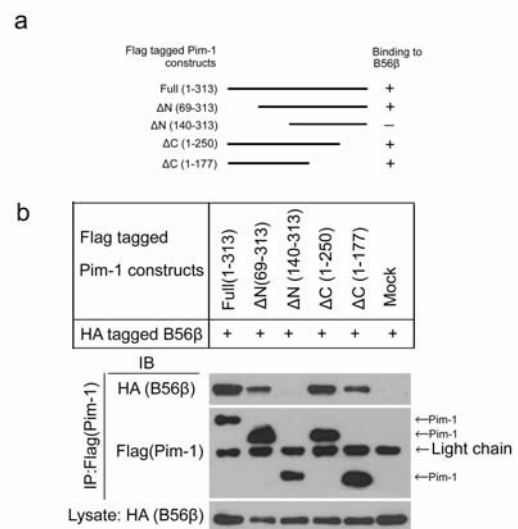
Task 3- The goal of this next task is understand how Pim-1 is degraded. To check whether Pim-1 might be regulated by ubiquination we transfected Pim along with an HA-

tagged ubiquitin vector. First, we demonstrated that Pim-1 is ubiquitinated so that the degradation of Pim-1 is clearly mediated by the proteasome. Interestingly, the levels of ubiquitination of Pim-1 are mediated by the presence of absence of PP2A B56beta protein. Knockdown of this subunit using RNAi clearly demonstrates that the ubiquitination of Pim-1 decreases (Figure 5) with inactivation of PP2A. Thus, PP2A controls the levels of Pim protein by regulating ubiquitination.

Figure 5 - Ubiquitinylated Pim-1 protein is decreased by B56β knockdown. HEK-293T cells were contransfected with CMV-HA-ubiquitin, pcDNA3/Flag-Pim-1, and shRNA expression vector [scrambled control or targeted to B56β] for 48 h. Cells were then maintained in DMEM containing 1% FBS with 1 μM Bortezomib for 6 h. Cells were harvested and lysates were divided for immunoprecipitaion with anti-Flag beads or for input detection. 5% of the lysates sample was used for input detection and 50% of the immunoprecipitation samples were analyzed by Western blotting with anti-ubiquitin and anti-Pim-1.

Secondly, we find that Pim-1 binds to the prolyl isomerase Pin-1. Pin-1 is an important enzyme that is necessary for the isomerization of proline residues. The binding of Pin-1 to Pim-1 appears to decrease the level of Pim-1 in the cells. To evaluate this possibility we transfected Pim-1 and Pin-1 into cells and asked whether they coimmunoprecipitate. The results of experiments demonstrating these points are seen in Figure 6.

Figure 6 The Pin1 Isomerase Associates with Pim-1 and B56β and facilitates Pim-1 degradation. (a) HEK-293T cells were contransfected with 1 μg CMV-HA-Pin1, and (or) 1 μg pcDNA3/Flag-Pim-1, 1 μg pCEP4HA-B56β, as indicated. Anti-Flag immunoprecipitations were carried out on cleared lysates. 5% percent of the extract was used to measure transfected protein levels while 50% of the immunoprecipitates were analyzed by Western blotting with anti-HA for B56β, Pin1, and anti-Flag for Pim-1. (b) Knockdown of Pin1 results in increased Pim-1 expression. (Upper panel)HEK-293T cells were contransfected with 0.5 μg HA/Pin1, and increasing amounts from 1 μg to 2 μg of shRNA-Pin1 (Sigma Mission shRNA, Cat No. TRCN0000001034). Cells were maintained in DMEM supplemented with 2% FBS for 72 h. Lysates were prepared and normalized for Western blotting. (Bottom panel) Cells were contransfected with 0.2 μg pcDNA3/Pim-1, and increasing amounts from 1 μg to 2 μg of shRNA-Pin1. Cells were maintained in DMEM supplemented with 2% FBS for 72 h.
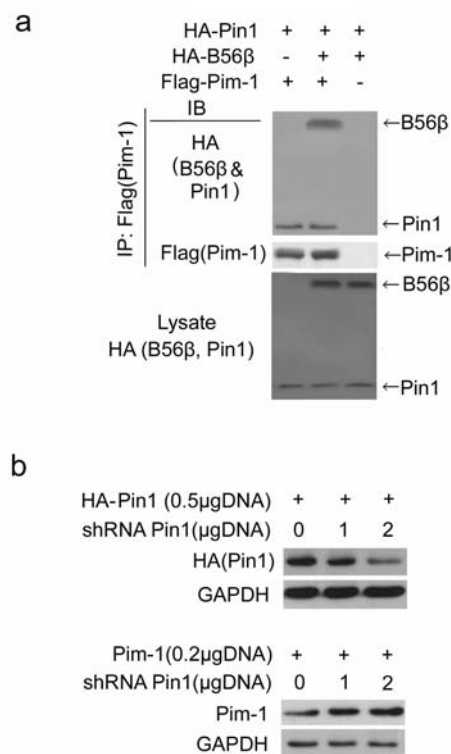
Task 4- The goal of this task is come up with a model that explains the control of the intracellular levels of Pim-1. Based on the results of Task 1-3 we have derived a model of how the level of Pim-1 protein may be controlled in prostate tumor cells. Lesions in any of these control mechanisms could elevate

6

the levels of Pim-1 protein. This mechanism suggests that Pin1 binds to Pim-1 isomerizing the protein. This leads to PP2A binding, dephosphorylation of Pim-1, and the ubiquitination of the Pim-1 protein. Degradation of Pim-1 then follows (see Figure 7 below). Clearly, a decrease in the activity of PP2A will lead to increases in Pim-1. The increase in Pim could be regulated in parallel to the c-myc protein, and help us to explain why both of these proteins are elevated in prostate cancer.

Figure 7 Hypothetical model of Pim-1 degradation by the proteasome mediated by Pin1 and PP2A.
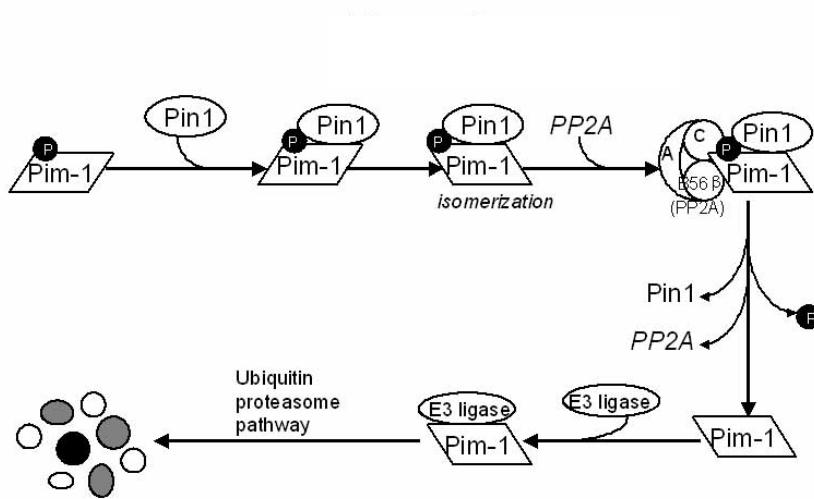
<u>Research Accomplishments</u>

- Increased PP2A activity negatively regulates Pim-1 protein levels

- PP2A dephosphorylates Pim-1 and decreases Pim-1 kinase activity

- The PP2A B56 beta subunit associates with Pim 1 in cells

- Knockdown of B56 beta increases Pim-1 protein expression

- B56beta affects the half-life and ubiquitinylation of Pim-1

- The Pin1 proplyl isomerase associates with Pim-1 in vivo and facilitates Pim-1 degradation.

- Knockdown of B56 beta increases cell viability in Pim containing but not negative cell lines

- A new model can be constructed to understand control of Pim levels.

Reportable Outcomes

1. Chen W.W., Chan, D.C. Donald, C., Lilly, M.B., and Kraft, A.S. Pim family kinases enhance tumor growth of prostate cancer cells. Molecular Cancer Res. 3(8): 443-551, 2005.

2. Ma, J., Arnold, H.K., Lilly, M.B., Sears, R.C., and Kraft, A.S. Negative regulation of Pim-1 protein kinase levels by the B56 beta subunit of PP2A. in press Oncogene

Conclusions

The results from year 1 of this proposal clearly demonstrated that Pim-1 makes prostate cancer grow faster and makes the tumors resistant to chemotherapy. The biochemical mechanism for this effect is suggested by the observation that the Pim protein kinase appeared to regulate the TOR pathway and inhibit the activity of PP2A. Inhibition of PP2A was felt to increase the activity of c-Myc. To understand in more depth how Pim-1 is functioning, we have focused on the regulation of Pim-1 by the PP2A phosphatase. We find that PP2A activity decreases both the level and activity of the Pim protein kinases. Okadaic acid and the SV40 small T antigen that are known modulators of PP2A inhibit this effect. Our results demonstrate that Pim-1 binds to the beta subunit of PP2A. Based on the observation that the Pin-1 proplyl-isomerase binds Pim-1 and decreases the level of this protein we have arrived at the following hypothesis. We believe that Pim-1 is phosphorylated, allowing the binding of Pin-1. Isomerization of the molecule then allows the interaction with PP2A B subunit. Pim-1 is then dephosphorylated and this then allows the molecule to be ubiquitinated and degraded. Since PP2A plays an essential role in regulating the TOR pathway, these observations connect growth factor signal transduction and Pim levels in prostate cancer.

Pim Family Kinases Enhance Tumor Growth of Prostate Cancer Cells

Wei Wei Chen,[1] Daniel C. Chan,[3] Carlton Donald,[2] Michael B. Lilly,[4] and Andrew S. Kraft[1]

[1]Hollings Cancer Center and [2]Department of Pathology, Medical University of South Carolina, Charleston, South Carolina; [3]Division of Medical Oncology, University of Colorado Health Sciences Center, Denver, Colorado; and [4]Center for Molecular Biology and Gene Therapy, Loma Linda University School of Medicine, Loma Linda, California

Abstract

Recent analyses indicate that the expression of the Pim-1 protein kinase is elevated in biopsies of prostate tumors. To identify the mechanism by which the Pim kinases may affect the growth of prostate tumors, we expressed Pim-1, Pim-2, or a kinase-dead Pim-2 protein in human PC3 prostate cancer cells. On implantation of the transfectants in nude mice, the growth of the cells expressing Pim-1 or Pim-2 was significantly faster than the growth of the control cells transfected with the neomycin-resistant gene or the kinase-dead Pim-2 protein. When grown in medium, the doubling time of the Pim-1 and Pim-2 transfectants was faster (0.75 days) than that of the control cells (1.28 days). We, therefore, examined the ability of Pim to control the phosphorylation of proteins that regulate protein synthesis. On growth factor starvation or rapamycin treatment, the Pim-1 and Pim-2 transfectants maintained their ability to phosphorylate 4E-BP1 and S6 kinase, although this phosphorylation did not occur in the control-transfected PC3 cells. We have found that the cellular levels of c-Myc were elevated in the Pim-1 and Pim-2 transfectants under these conditions. The Pim-1 and Pim-2 transfectants have lower levels of serine/threonine protein phosphatase 2A (PP2A) activity and the α- and β-subunit B56γ of the PP2A phosphatase do not coimmunoprecipitate in these cells. Thus, the effects of Pim on PP2A activity may mediate the levels of c-Myc and the phosphorylation of proteins needed for increased protein synthesis. Both of these changes could have a significant impact on tumor growth. (Mol Cancer Res 2005;3(8):443–51)

Introduction

Prostate cancer is the most common malignancy diagnosed in men in the United States. Recent evidence suggests that the Pim family of protein kinases may play a role in the development or progression of this cancer. The report of elevated levels of *pim-1* in human prostate tumors on cDNA and microarray analysis implicates the Pim family of serine/threonine kinases in the progression of human prostate tumors (1). In transgenic animal models, Pim-1 expression has been shown to be elevated in prostate tumors that are caused by overexpression of the c-*myc* oncogene (2). Although the Pim kinases have been identified as oncogenes in transgenic models, by themselves they are only weakly transforming. They have, however, been shown to greatly enhance the ability of c-*myc* gene to induce lymphomas (3, 4). The regulation of the expression of the Pim kinases and their function has been analyzed extensively in hematopoietic cells. It has been shown that the levels of Pim-1 and Pim-2 protein can be regulated by the addition of granulocyte-macrophage colony stimulating factor, interleukin-3, and interleukin-7 to normal hematopoietic cells (5) through activation of the Janus-activated kinase/signal transducers and activators of transcription pathway (6). In addition, tumor necrosis factor and Toll-like receptor ligands have been shown to induce the expression of Pim-1 (7-9). Although the overexpression of either Pim-1 or Pim-2 factor–dependent hematopoietic cells makes them resistant to apoptosis induced by interleukin-3 withdrawal (10-12), kinase-dead mutants of *pim-1* do not protect against apoptosis (13, 14). It also has been shown that the BH3 protein BAD can be phosphorylated by Pim-1 and Pim-2 and protect against apoptosis (12, 15).

Other previously described phosphorylation targets include HP-1 (16), cdc 25A phosphatase (17), and SOCS-1 (18). More recently, Pim-2 has been shown to phosphorylate the ribosomal protein 4E-BP1, causing it to dissociate from eIF-4E, which may affect protein synthesis (11). These data indicated that the ability of these protein kinases to inhibit cell death and regulate tumorigenesis is likely controlled by phosphorylation of specific target proteins. To explore the role of Pim in human prostate cancer, we have established overexpressor cell lines in PC3 cells.

Results and Discussion

To investigate the role of the Pim protein kinase family in controlling prostate cancer growth, we have created PC3 human prostate cancer cell lines that overexpress either murine Pim-1

A

B

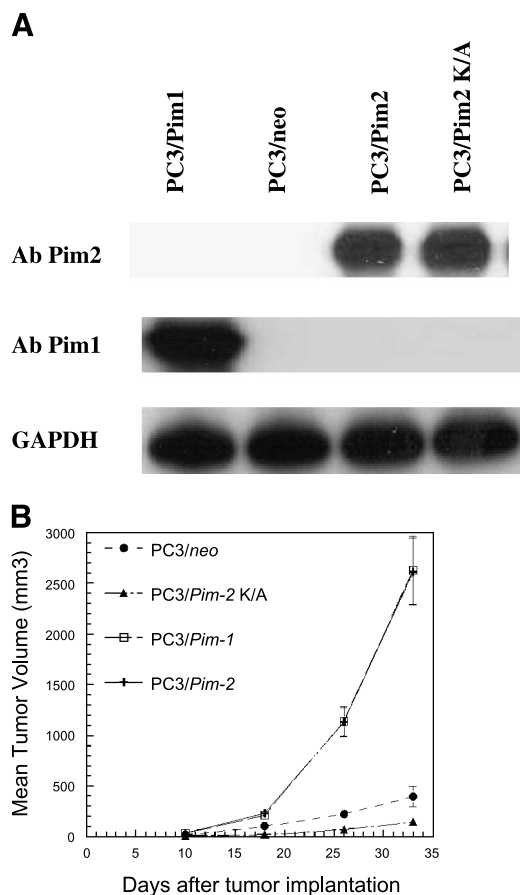**FIGURE 1.** Pim kinases enhance the growth PC3 prostate tumor cells implanted in BALB/c *nu/nu* mice. **A.** Cell lines were established from PC3 cells that had been transfected with Pim-1, Pim-2, a kinase-dead mutation of Pim-2 (Pim-2 K/A), or the neomycin resistance gene. The expression of Pim-1 and Pim-2 in the cell lines is equivalent as indicated by Western blot analysis of cell lysates on 10% SDS-PAGE using monoclonal antibodies (12) to probe the blotted polyvinylidene difluoride membrane. The PC3/*neo* transfectant does not express levels of Pim-1 or Pim-2 that are detectable using this technique. **B.** PC3/*neo*, PC3/Pim-1, PC3/Pim-2, and PC3/Pim-2 K/A cells (2 × 10^6 per xenograft) were injected subcutaneously into the flanks of BALB/c *nu/nu* mice with five mice in each group with four tumors injected into each mouse. The tumor volume was calculated from caliper measurements in two dimensions at the indicated time points. Points, mean of 20 measurements (four tumors per mouse and five mice per group); bars, SD. The growth of Pim-1 – and Pim-2 –containing tumors are shown as overlapping lines.

or Pim-2. At the amino acid level, human and mouse Pim-1 are 89.9% identical, whereas the Pim-2s are 86.4% identical. We have created two control cell lines by transfecting PC3 cells with either a kinase-inactive form of Pim-2 that was generated by mutating Lys^{120} to alanine using the Quik Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) or the neomycin-resistance gene (Fig. 1A). These are called PC3/Pim-1, PC3/Pim-2, PC3/Pim-2 K/A, and PC3/*neo*, respectively. To evaluate the effect of increasing Pim levels on tumor growth, the cell lines were injected subcutaneously into BALB/c *nu/nu* mice. Both PC3/Pim-1 – and PC3/Pim-2 –derived tumors (overlapping growth curves in Fig. 1B) grew significantly faster than PC3/*neo* –derived tumors when comparing tumor size on day 33 (paired Student's *t* test PC3/*neo* compared with

PC3/Pim-1, $P < 1.36 \times 10^{-6}$; PC3/*neo* compared with PC3/Pim-2, $P < 3.33 \times 10^{-6}$). Interestingly, the kinase-dead Pim-2 – transfected cells did not grow well as tumors (Fig. 1B). It is possible that kinase-dead Pim acts as a dominant-negative mutation inhibiting protein synthesis or phosphorylation of specific substrates and blocking tumor growth.

To examine whether Pim stimulates the growth rate of PC3 cells, we measured the growth rate of the Pim transfectants in tissue culture medium by plating the cells at a low density and counting cell numbers over time (Fig. 2A). Under these conditions, both the PC3/Pim-1 and PC3/Pim-2 transfectants had a doubling time of 0.75 days, whereas the doubling time of the PC3/*neo* and PC3/Pim-2 K/A cells was almost twice as long at 1.28 and 1.20 days, respectively. 4′,6-Diamidino-2-phenylindole staining of the cells disclosed that there was no significant difference in the rate of apoptosis between the different transfectants. We then examined the effect of Pim on cell growth under nutrient-limiting conditions; we starved cells of growth factors or treated them with rapamycin, which is known to block cells in the G$_1$ phase of the cell cycle (19). As expected, under these conditions, the PC3/*neo* and PC3/Pim-2 K/A cells were blocked in the G$_1$ phase of the cell cycle; however, at least over the short time period of this assay, the cell cycle distribution of the PC3/Pim transfectants was not affected significantly (Fig. 2B).

As Pim-2 has been shown to regulate the phosphorylation of proteins that control protein synthesis in hematopoietic cells (11), we investigated whether a similar mechanism might be operative in prostate tumor cells by determining the ability of overexpression of the Pim protein kinases to enhance the phosphorylation of 4E-BP1 and S6 kinase. The cell lines were either exposed to serum deprivation and nutrient starvation, treated with rapamycin, or treated with the two regimens in combination. The activity of target of rapamycin (TOR) is inhibited by the dose of rapamycin used in these experiments (20).

4E-BP1 is highly phosphorylated on Thr^{37}, Thr^{46}, Thr^{70}, Ser^{65}, Ser^{83}, Ser^{101}, and Ser^{112}. A two-stage mechanism of phosphorylation has been proposed in which Thr^{37} and Thr^{46} are phosphorylated and then allow phosphorylations to occur on Ser^{65} and Thr^{70} (21). The latter two phosphorylations are essential for inhibiting the binding of 4E-BP-1 to eIF4E (22). A number of the phosphorylations may be minor, including Ser^{83}, or constitutive, e.g., Ser^{101} and Ser^{112} (23). More highly phosphorylated 4E-BP1 has been associated with the malignant phenotype in human mammary epithelial cells (24). On SDS polyacrylamide gels, the more highly phosphorylated 4E-BP1 has been labeled γ, whereas the least phosphorylated form is denoted α (Fig. 3A; ref. 24). Multiple protein kinase pathways are thought to play a role in regulating phosphorylation of 4E-BP1. TOR activity may be required as a priming event stimulating the phosphorylation of Thr^{37} and Thr^{46}. The protein kinases that phosphorylate Ser^{65} and Thr^{70} have not been fully identified. It has been suggested that these phosphorylations may be downstream of the extracellular signal-regulated kinase or protein kinase B and directly regulated by insulin and phorbol esters (25, 26). We show that the addition of rapamycin, and to a lesser extent serum starvation, to wild-type PC3 cells markedly diminishes the phosphorylation of Thr^{37}, Thr^{46}, and Thr^{70} (Fig. 3A). In Pim-containing cells

growing in serum, the presence of either Pim-1 or Pim-2 seems to enhance the phosphorylation of 4E-BP1. In Pim-2– containing cells, neither serum starvation alone nor in combination with rapamycin decreases this phosphorylation. In

Pim-1–containing PC3 cells, there is a clear decrease in phosphorylation with these treatments although there is no decrease to baseline. It is possible that Pim-1 may not function identically to Pim-2. We have found that transfected Pim-1 is

%	PC3	PC3 -S	PC3 -S+Ra	Pim-1	Pim-1 -S	Pim-1 -S+Ra	Pim-2	Pim-2 -S	Pim-2 -S+Ra	K/A	K/A -S	K/A -S+Ra
G1	41.65	66.67	80.11	44.23	51.74	46.79	50.12	42.85	53.67	40.81	72.25	82.21
G2/M	27.73	19.05	11.79	24.5	15.34	20.86	25.58	26.75	22.71	20.63	17.42	7.38
S	30.62	14.28	8.1	31.25	32.92	32.35	24.3	30.4	23.62	38.56	10.33	10.4
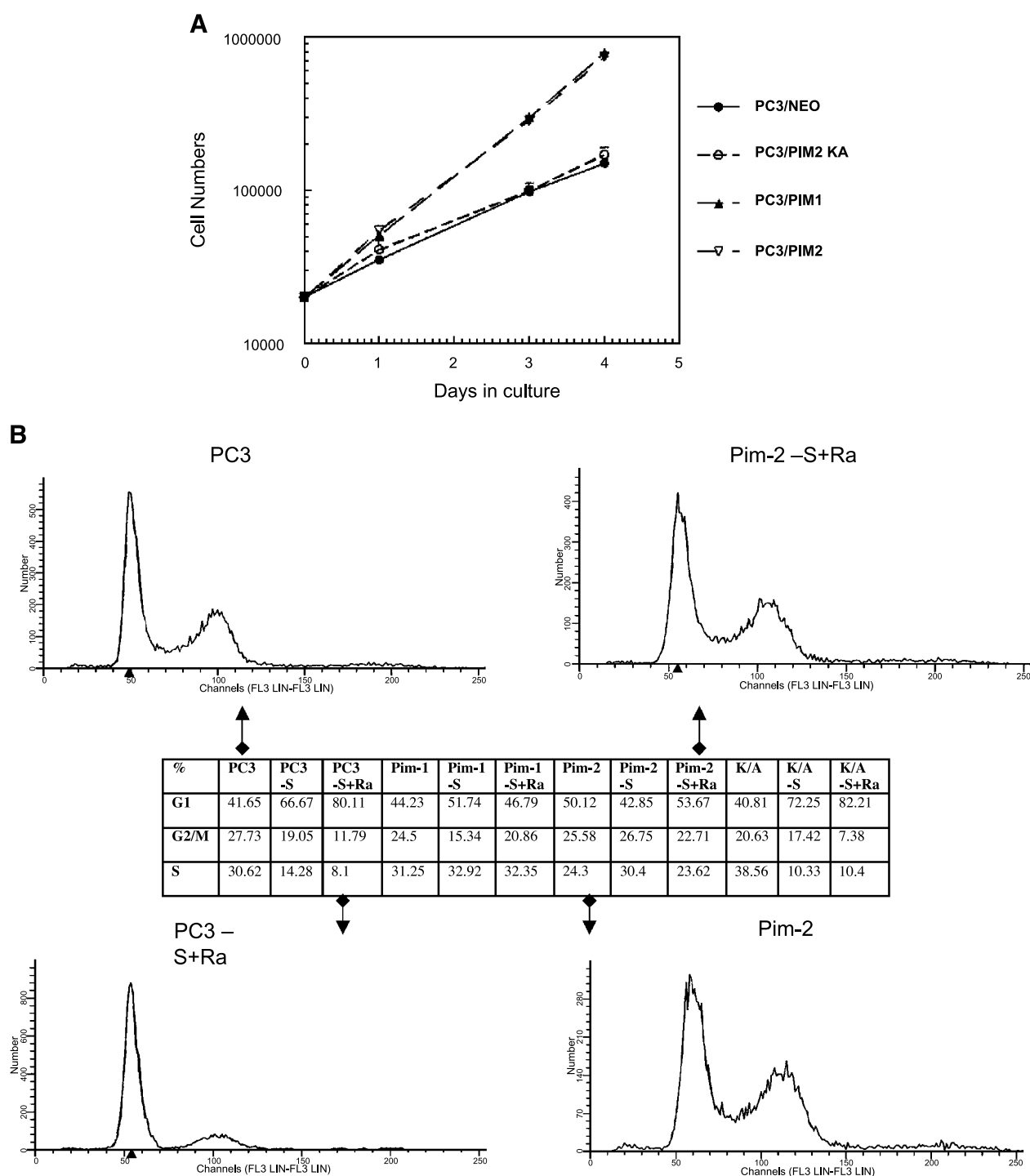
FIGURE 2. Expression of Pim-1 or Pim-2 enhances the doubling time of PC3 prostate tumor cells in culture. **A.** The PC3 cells were plated at low density in triplicate wells and the cells harvested and counted at the indicated time points. Points, average of triplicate determinations; bars, SD. **B.** PC3 cell lines were starved of serum (−S) for 24 hours then either treated with rapamycin (80 nmol/L) for an additional 24 hours (+Ra) or maintained under serum-free conditions. The cells were then trypsinized, fixed in 70% ethanol, stained with propidium iodide, and subjected to DNA histogram analysis by fluorescence-activated cell sorting. The results presented are the average of triplicate determinations. However, cell cycle histograms representing individual experiments are shown.

A

B

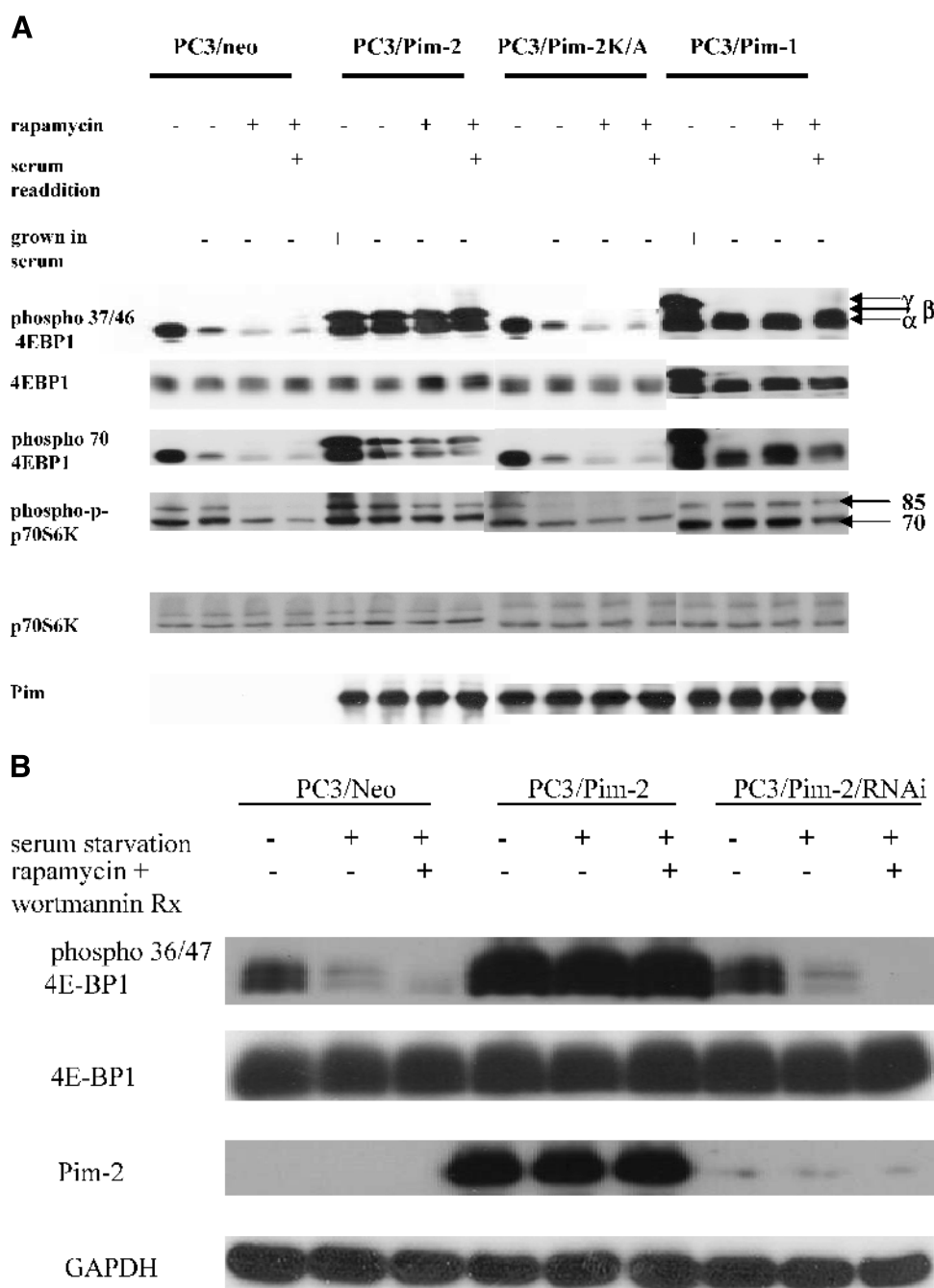**FIGURE 3.** Expression of Pim-1 or Pim-2 kinase maintains phosphorylation of key enzymes on serum starvation or treatment with rapamycin. **A.** PC3 cell lines containing either the neomycin-resistance gene, Pim-1, Pim-2, or Pim-2 K/A, were grown in serum, or after serum starvation for 24 hours, fresh serum-free medium was added followed by an additional 24 hours incubation with or without rapamycin (80 nmol/L). To examine the ability of serum to overcome inhibition by rapamycin (80 nmol/L), a portion of the serum-starved rapamycin (80 nmol/L)−treated cells was incubated with 20% FCS for 30 minutes before homogenization. Cellular extracts were electrophoresed on a 12% SDS-PAGE gel and then transferred to polyvinylidene difluoride membranes. Additional extracts were run to probe for S6 kinase isoforms. Arrow, phosphorylated p70 and p85 S6K protein; arrows, multiple forms of 4E-BP1doublet points at the multiple phosphorylated forms of 4E-BP1. **B.** RNA interference was used to validate the role of Pim-2 in controlling 4E-BP1 phosphorylation. PC3 cells expressing Pim-2 were transfected with an RNA interference that knocks down Pim-2 protein levels. Cells were then starved of serum for 24 hours followed by treatment with rapamycin (80 nmol/L) and wortmannin (20 nmol/L) for an additional 24 hours. Extracts were run on SDS-PAGE gels and immunoblotted with antibodies to the phosphorylated form of 4E-BP1, 4E-BP1, Pim-2, and GAPDH.

largely located in the nucleus (27), whereas Pim-2 is thought to be cytoplasmic. Pim-1 could function in the nucleus to effect the transcription of proteins that modify the function of TOR, e.g., rictor and raptor, or specific phosphatases that regulate these proteins. Pim-2 could work directly in the cytoplasm to phosphorylate 4E-BP1 or regulate the activity of specific phosphatases.

A number of possibilities may explain these results. The Pim protein kinases may be phosphorylating Thr^{37} and Thr^{46} directly, mimicking TOR and enabling the phosphorylation of Ser^{65} and Thr^{70} by additional protein kinases. Or, Pim may be

responsible for Ser^{65} and Thr^{70} phosphorylation. Other possibilities to explain Pim-2 protein kinase activity include regulation of the rapamycin-insensitive TOR complex bound to the protein rictor (28) or modulation of the protein phosphatase 2A (PP2A) activity that is an important regulatory protein in the TOR pathway in yeast (29). The S6 kinase message encodes two isoforms, p70 and p85 (30). Like 4E-BP1, S6 kinase is activated by a two-step mechanism by which TOR phosphorylates multiple sites (Ser^{411}, Ser^{418}, Ser^{424}, and Thr^{421}), which is then followed by an activating phosphorylation (Thr^{229}) by PKD1 protein kinase (31). We find that amino acid starvation

and rapamycin treatment markedly decreases the phosphorylation of both p70 and p85 S6 kinases (Fig. 3A). In contrast, Pim protein kinases maintain these two kinases in the phosphorylated and active form. The activity of the Pim kinases may be explained by the ability of these enzymes to phosphorylate and activate a subset of TOR protein complexes, phosphorylate S6 kinase directly, and regulate the dephosphorylation of this protein kinase or the activity and specificity of PKD1.

Using RNA interference directed at Pim-2 that has been validated in other cell lines, it is shown that the knockdown of Pim-2 protein levels in PC3 Pim-2 cells markedly inhibits the phosphorylation of 4E-BP1 (Fig. 3B; ref. 11). However, it had no effect on the total cellular levels of 4E-BP1. This knockdown occurs in the presence of rapamycin and wortmannin, where Pim seems to be regulating 4E-BP1 phosphorylation. In contrast, this

small interfering RNA treatment had no effect on the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in these cells. These data confirm that Pim-2 can modulate the phosphorylation of proteins that play a crucial role in controlling protein synthesis in prostate cancer. We also show the ability of overexpressed Pim-1 compared with Pim-2 to modulate the phosphorylation in a similar if not identical fashion. This regulation of protein synthesis may contribute significantly to the ability of Pim protein kinases to enhance tumor growth.

Cap-dependent translation controls the level of multiple proteins, including c-Myc, a protein that seems to collaborate with Pim (32, 33). Under the conditions described above, we evaluated the cellular levels of c-Myc and found that the level of this protein is higher in the Pim-1 and Pim-2 transfectants than in control PC3/*neo* cells (Fig. 4A). Growth factor
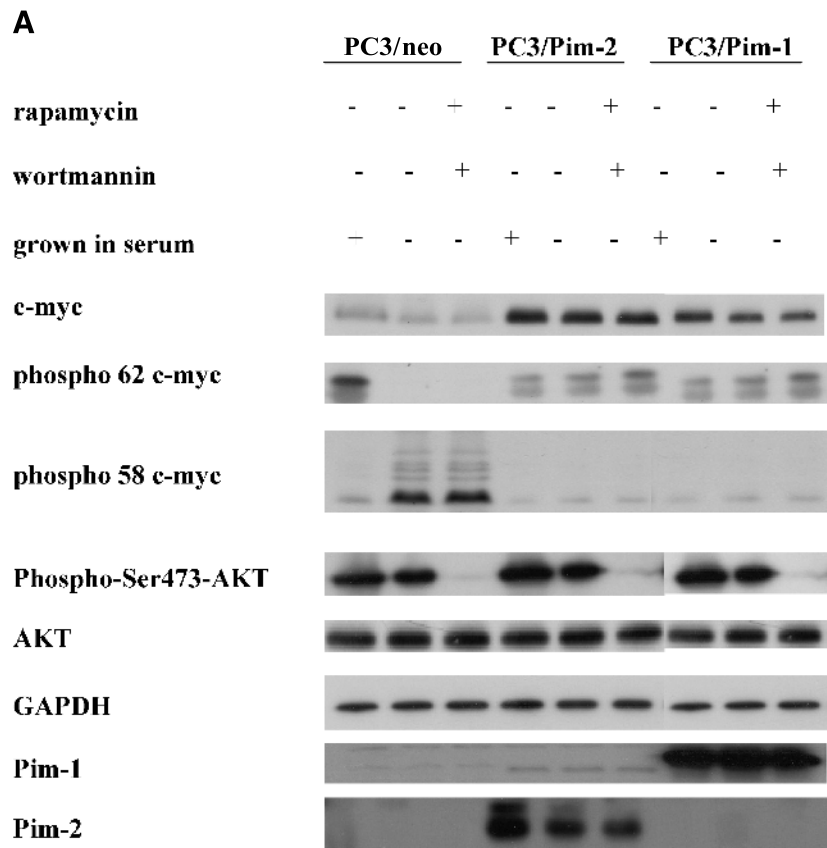

FIGURE 4. Regulation of c-Myc levels in Pim-containing PC3 cells. **A.** To evaluate c-Myc levels, PC3/*neo*, PC3/Pim-1, and PC3/Pim-2 were grown in RPMI, including 10% fetal bovine serum, then serum-starved for 24 hours and treated with rapamycin (80 nmol/L) and wortmannin (20 nmol/L) for an additional 24 hours before harvesting. Extracts were run on SDS-PAGE gels. The membrane was stripped and probed with antibodies specific for c-Myc, phospho-Ser[62], phospho-Thr[58], phospho-Ser[473] AKT, AKT, and GAPDH, a loading control. **B.** To measure the level of *c-Myc* mRNA in PC3 cells, mRNA was extracted as described in Materials and Methods and subjected to quantitative reverse transcription-PCR. The level of *c-myc* mRNA was compared with GAPDH and expressed as a ratio for each sample. The experiment was repeated with three individual RNA samples and was done in triplicate. Columns, mean; bars, SD.
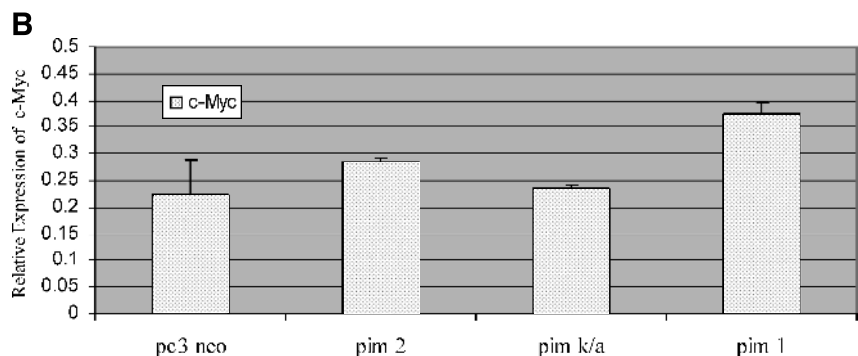
starvation or rapamycin and wortmannin treatment decreased the level of c-Myc in control cells. Likewise, these treatments inactivated the endogenous AKT phosphorylation that is elevated in PC3 cells. These manipulations had little effect on the level of c-Myc in the Pim-containing cells. Phosphorylation of c-Myc on Ser[62] by the action of the extracellular signal-regulated kinase pathway stabilizes the protein, whereas GSK-3β phosphorylation of Thr[58] targets the protein to ubiquitin-mediated degradation (34, 35). Serum starvation of PC3/*neo* cells or treatment with wortmannin and rapamycin increased phosphorylation of Thr[58] while decreasing phosphorylation of Ser[62]. In the Pim-1 and Pim-2 transfectants, these treatments had no significant effect and Ser[62] remained phosphorylated. The combined treatment with rapamycin and wortmannin clearly inhibited the activity of the AKT protein kinase in all cell lines (Fig. 4A). It has been shown previously that c-Myc in which Ser[62] is phosphorylated, but not unphosphorylated c-Myc, functions to transform normal human fibroblasts transfected with human telomerase and Ras[G12V] and is responsible for activation of the E2F promoter (36). Activated c-Myc can increase the transcription of eIF-4E, further enhancing protein synthesis (37-39). Thus, it is possible that modulation of the phosphorylation and, hence, the levels of c-Myc by Pim may be important in its mechanism of action and the regulation of tumor cell growth.

To evaluate the possibility that the mechanism by which Pim regulates c-Myc protein levels is through increasing c-Myc mRNA, we did quantitative PCR. The levels of c-Myc were normalized to GAPDH and the experiment was repeated on three independent samples. The results show that control cells, Pim-2, and Pim-2 K/A cells have very similar levels of c-Myc mRNA (Fig. 4B). In contrast, we find that the Pim-1 cells seem to have increased levels of c-Myc mRNA when compared with the other three cell lines. Student's *t* test comparing the levels of c-Myc with the control cells shows a *P* value of 0.03, suggesting only modest statistical difference. It has been suggested that the biological activities of Pim-1 and Pim-2 are different (11). Pim-2 functions in the cytoplasm and stimulates the Cot kinase to enhance the activity of nuclear factor-κB (40). In contrast, we have found that Pim-1, when transfected into 293T cells or in stained human Burkitt's lymphoma cells, is located in the nucleus (27). Thus, it is possible that Pim-1 could regulate c-Myc mRNA levels in a different manner than Pim-2.

The phosphorylation of serine PP2A results in the dephosphorylation of c-Myc on Ser[62], thereby allowing its Thr[58]-directed protein ubiquination and degradation (36). The degradation of c-Myc blocks its cell cycle– and growth-promoting activity, inhibiting its ability to regulate transcription. The serine phosphatases also have been shown to play an important role in regulating protein synthesis in yeast (41). Pim has been shown to coimmunoprecipitate with the catalytic subunit of PP2A (42), suggesting that Pim might regulate the activity of this phosphatase. Using a colorimetric assay for PP2A that was validated by demonstrating its inhibition with okadaic acid and sodium fluoride (Fig. 5A), we measured PP2A activity in the four PC3 cell lines (Fig. 5A). This experiment showed that the Pim-1 and Pim-2 transfectants exhibited a significantly lower level of PP2A activity than the PC3/*neo*
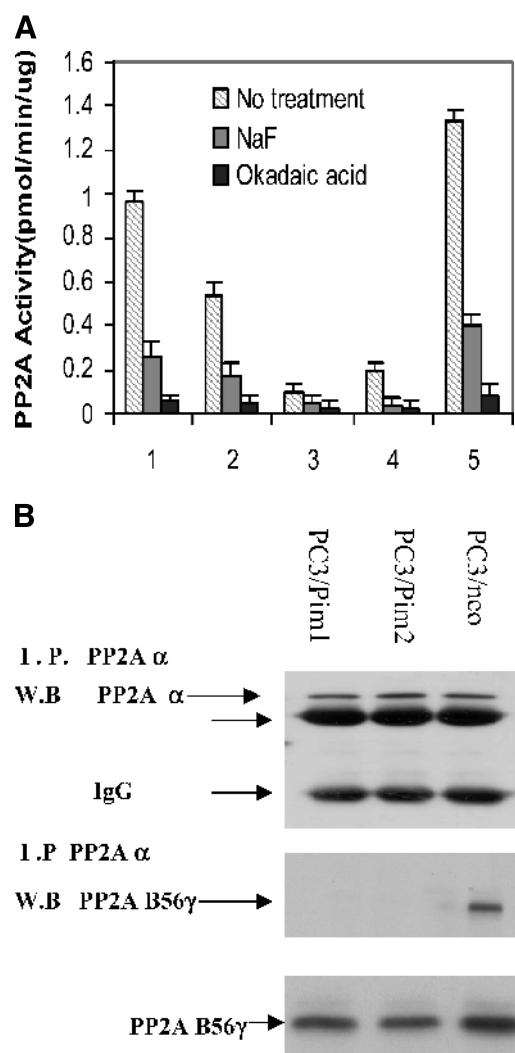

FIGURE 5. Expression of Pim-1 or Pim-2 regulates PP2A activity in PC3 prostate tumor cells. **A.** Cells (2.5 × 10⁶) were harvested in Tris-HCl–buffered saline, centrifuged, and then sonicated in 500 μL buffer [10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, and 0.02% sodium azide]. The phosphatase assay was carried out as described in Materials and Methods. As controls, either sodium fluoride (50 mmol/L) or okadaic acid (5 μmol/L) was added to cell extracts before beginning the assay. Columns, mean of triplicate determinations; bars, SD. 1, PC3/neo; 2, PC3/Pim-2 K/A; 3, PC3/Pim-2; 4, PC3/Pim-1; 5, PP2A catalytic subunit. **B.** 1 × 10⁶ cells were lysed in 50 mmol/L Tris-HCl (pH 7.4); 150 mmol/L NaCl; 1 mmol/L EDTA; 1 mmol/L phenylmethylsulfonyl fluoride; and 1 μg/mL each of aprotinin, leupeptin, and pepstatin. The extracts were immunoprecipitated with an antibody to the α-subunit of PP2A. The immunoprecipitates were run on a 10% SDS-PAGE gel, transferred to polyvinylidene difluoride membranes, and probed with antibodies to the α-subunit of PP2A or an antibody to the β-subunit B56γ. Arrows, α- and β-subunits and the IgG light and heavy chains.

control cells. Interestingly, the PC3/Pim-2 K/A cells had a slightly lower level of phosphatase activity than PC3/*neo* controls, possibly suggesting that this protein could interact with PP2A in the absence of kinase activity. Recent evidence (43) has shown that polyoma small-T antigen inhibits PP2A activity by causing the dissociation of the catalytic and regulatory subunits of this phosphatase. To investigate this possibility, we immunoprecipitated the PP2A α-subunit and

then Western blotted with an antibody to the β-component of the complex, B56γ. Our results show that the overexpression of Pim-1 or Pim-2 compared with the PC3/neo cells caused apparent disassociation of these two subunits. However, the total levels of B56γ do not change in these cells (Fig. 5B). This dissociation could function to inhibit PP2A activity, and thus indirectly regulate the phosphorylation of c-Myc as seen in Fig. 4A. By modulating c-Myc phosphorylation and thus its degradation, Pim protein kinases can function to enhance the activity of c-Myc and potentially to stimulate transit through the cell cycle.

The TOR pathway and phosphorylation of 4E-BP1 are critical to the growth of tumors (26, 44). Multiple enzyme abnormalities in tumor cells affect this pathway, including increased activity of AKT (45, 46) and mutation of TSC1 and TSC2 (47, 48). Normally, the phosphorylation of the 4E-BP1 protein and the p70S6 kinase is stimulated by the AKT protein kinase through a TOR-dependent pathway (19, 49). The phosphorylation of p70S6 kinase causes phosphorylation of the S6 protein and stimulates protein synthesis. Phosphorylation of 4E-BP1 on Thr[37] and Thr[46] potentiates the further phosphorylation of 4E-BP1 on Ser[70], dissociating this protein from eIF-4E. Free eIF-4E stimulates Cap-dependent protein translation of a large number of proteins, including c-Myc. Pim family members can phosphorylate the sequence RRRLS/T (50), which is similar to that modified by AKT, RXRXXS/T (51), suggesting that they could affect similar pathways. However, the observation that rapamycin blocked AKT-mediated TOR phosphorylation, but not Pim-mediated phosphorylation of 4E-BP1, suggests that the mechanisms by which Pim affect the pathway differ from AKT. It may be inferred, however, that the effect of Pim on PP2A could also be crucial in regulating protein synthesis.

There has been recent interest in the development of rapamycin analogues as treatment regimens for tumors in which PTEN is deleted (52-56) or as drugs that could sensitize tumors to other chemotherapeutic agents (20). Clearly, tumors that overexpress Pim would be resistant to both of these approaches. Knowledge of the level of overexpression of Pim in tumors may help in planning chemotherapy regimens. Additionally, targeting the Pim kinases with small molecule inhibitors may play a critical role in cancer therapy.

Materials and Methods

Materials

Rapamycin, wortmannin, and antibodies to wild-type and phosphorylated forms of 4E-BP1, phospho-Ser[473] AKT, AKT, and p70S6K were purchased from Cell Signaling Technologies (Beverly, MA). Okadaic acid was obtained from Invitrogen (Carlsbad, CA). Monoclonal Pim-2 and Pim-1 antibody were generated by immunization with a 20–amino acid peptide corresponding to the carboxyl terminus of the Pim-2 and Pim-1 protein. Antibodies were purified by affinity chromatography using peptides covalently liked to 4B Sepharose (Amersham, Piscataway, NJ). Anti-flag M2 was purchased from Eastman Kodak Company (New Haven, CT). Additional antibodies used include c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Ser[62] (Abcam, Cambridgeshire, United Kingdom), phospho-Thr[58] (Cell Signaling), α-subunit of PP2A (6F9;

Covance, Berkeley, CA), an antibody to the β-subunit B56γ a gift of Dr. William Hahn (Harvard Medical School, Boston, MA), and GAPDH (Chemicon, Temecula, CA), a loading control.

Plasmids

The murine *pim-1* and *pim-2* cDNAs were PCR amplified using primers with *Eco*RI and *Xho*I restriction site at their ends and cloned into the PCDNA.3.1 expression vector (Invitrogen). A kinase-dead mutant form of pim-2 expression vector (*pimK/A*) was generated by mutating Lys[120] to alanine using the Quik Change Site-Directed Mutagenesis kit (Stratagene).

Cell Culture and Transfection

PC3 cell lines were maintained in RPMI 1640 containing 10% FCS penicillin/streptomycin. PC3 cells were transfected using Effectene (Qiagen, Valencia, CA) according to the instructions of the manufacturer. For derivation of cell lines after 48 hours, the culture medium was switched to the same medium supplemented with 1,000 μg/mL Geneticin. For rapamycin treatment of stable and transiently transfected cells, all cell lines were first placed in serum-free medium for 24 hours and then fresh serum-free medium was added before addition of 80 nmol/L rapamycin.

Western Blot Analysis

Cell extracts were prepared by lysing cells in a buffer containing 20 mmol/L Tris (pH 7.5),150 mmol/L NaCl, 1 mmol/L EDTA,1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium PPi,1 mmol/L β-glycerolphosphate, 0.5% NP40, 1 mmol/L Na$_3$VO$_4$, 1 μg/mL leupeptin, and protease inhibitor cocktail (Sigma Chemical, St. Louis, MO). The lysates were incubated on ice for 15 minutes followed by centrifugation for 10 minutes at 4°C. To the supernatant was added with 5× Ficoll and the mixture was heated at 100°C for 5 minutes. The cell extracts were resolved by 12% SDS-PAGE and then transferred to Immobilon-P membrane filters (Millipore, Bedford, MA).

Cell Cycle Analysis

Twenty-four hours after culturing prostate cancer cell lines in normal medium, they were transferred to serum-free medium and cultured for 24 additional hours. The tumor cells were then treated with 80 nmol/L rapamycin or DMSO for 18 hours and subjected to propidium iodide staining. The percentage of cells in different phases of the cycle was determined by fluorescence-activated cell sorting analysis.

Tumor Growth Curve

Subcutaneous tumor xenografts were established in nude mice by injecting tumor cells ($2 \times 10^{+6}$) subcutaneously in four locations on each mouse. Seven days after transplantation, tumor measurements were begun in a blinded fashion with five mice per group or 20 total tumors.

Phosphatase Assay

Cells (2.5×10^6) were harvested in Tris-HCl–buffered saline, centrifuged, and then sonicated in 500 μL buffer

[10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, and 0.02% sodium azide]. A 250 μL aliquot of the homogenate was applied to a G25 column to decrease the levels of free phosphate, and the flow through subjected to a PP2A colorimetric assay following the instructions of the manufacturer (Promega, Madison WI) and using the PP2A-specific buffer. As controls, either sodium fluoride (50 mmol/L) or okadaic acid (5 μmol/L) was added to cell extracts before beginning the assay.

RNA Interference

A short interfering RNA for Pim-2 (5′-GGGATAGATGGA-CATCTGTTGAA-3′) that had been previously validated (11) was purchased from Ambion (Austin, TX) and transfected into PC3 cells at a concentration of 100 pmol using LipofectAMINE 2000 (Invitrogen). Thirty-six hours later, the serum was withdrawn for 24 hours and then rapamycin (80 nmol/L) and wortmannin (20 nmol/L) were added for an additional 48 hours.

RNA Isolation and Quantitative Reverse Transcription-PCR

Total RNA (0.5 μg per reaction) was reverse transcribed into cDNA utilizing random primers (Promega). AMV Reverse Transcriptase II enzyme (500 units per reaction; Promega) was used for first-strand synthesis and Tfl DNA Polymerase for second-strand synthesis (500 units per reaction; Promega) as per the protocol of the manufacturer. In each case, 50 pg of cDNA was used per ensuing PCR reaction. Two-step quantitative reverse transcription-PCR was done on cDNA generated using the MultiScribe Reverse Transcriptase from the TaqMan Reverse Transcription System and the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA).

The primer pair for c-Myc was generated from the published c-Myc sequence (accession no. NM_002467). Forty cycles of PCR were done under standard conditions using an annealing temperature of 56°C. In addition, *GAPDH* was amplified as a housekeeping gene to normalize the initial content of total cDNA. Here, c-Myc expression was calculated as the relative expression ratio between c-Myc and GAPDH and was compared for each condition. As a negative control, quantitative reverse transcription-PCR reactions without cDNA template were also done. All reactions were run thrice in triplicate.

References
1. Dhanasekaran SM, Barrette TR, Ghosh D, et al. Delineation of prognostic biomarkers in prostate cancer. Nature 2001;412:822–6.

2. Ellwood-Yen K, Graeber TG, Wongvipat J, et al. Myc-driven murine prostate cancer shares molecular features with human prostate tumors. Cancer Cell 2003;4:223–38.

3. van Lohuizen M, Verbeek S, Krimpenfort P, et al. Predisposition to lymphomagenesis in *pim-1* transgenic mice: cooperation with c-*myc* and N-*myc* in murine leukemia virus-induced tumors. Cell 1989;56:673–82.

4. van Lohuizen M, Verbeek S, Scheijen B, Wientjens E, van der Gulden H, Berns A. Identification of cooperating oncogenes in E mu-myc transgenic mice by provirus tagging. Cell 1991;65:737–52.

5. Lilly M, Le T, Holland P, Hendrickson SL. Sustained expression of the pim-1 kinase is specifically induced in myeloid cells by cytokines whose receptors are structurally related. Oncogene 1992;7:727–32.

6. Sakai I, Kraft AS. The kinase domain of Jak2 mediates induction of bcl-2 and delays cell death in hematopoietic cells. J Biol Chem 1997;272:12350–8.

7. Saito Y, Tada H, Nazarea M, Honjo T. Interleukin 2 and tumor necrosis factor α are complementary for proliferation of the hematopoietic stem cell line LyD9. Growth Factors 1992;7:297–303.

8. Ishibashi Y, Maita H, Yano M, et al. Pim-1 translocates sorting nexin 6/TRAF4-associated factor 2 from cytoplasm to nucleus. FEBS Lett 2001;506:33–8.

9. Xu LG, Wu M, Hu J, Zhai Z, Shu HB. Identification of downstream genes up-regulated by the tumor necrosis factor family member TALL-1. J Leukoc Biol 2002;72:410–6.

10. Lilly M, Kraft A. Enforced expression of the M_r 33,000 Pim-1 kinase enhances factor-independent survival and inhibits apoptosis in murine myeloid cells. Cancer Res 1997;57:5348–55.

11. Fox CJ, Hammerman PS, Cinalli RM, Master SR, Chodosh LA, Thompson CB. The serine/threonine kinase Pim-2 is a transcriptionally regulated apoptotic inhibitor. Genes Dev 2003;17:1841–54.

12. Yan B, Zemskova M, Holder S, et al. The PIM-2 kinase phosphorylates BAD on serine 112 and reverses BAD-induced cell death. J Biol Chem 2003;278:45358–67.

13. Lilly M, Sandholm J, Cooper JJ, Koskinen PJ, Kraft A. The PIM-1 serine kinase prolongs survival and inhibits apoptosis-related mitochondrial dysfunction in part through a bcl-2-dependent pathway. Oncogene 1999;18:4022–31.

14. Shirogane T, Fukada T, Muller JM, Shima DT, Hibi M, Hirano T. Synergistic roles for Pim-1 and c-Myc in STAT3-mediated cell cycle progression and antiapoptosis. Immunity 1999;11:709–19.

15. Aho TL, Sandholm J, Peltola KJ, Mankonen HP, Lilly M, Koskinen PJ. Pim-1 kinase promotes inactivation of the pro-apoptotic Bad protein by phosphorylating it on the Ser^{112} gatekeeper site. FEBS Lett 2004;571:43–9.

16. Koike N, Maita H, Taira T, Ariga H, Iguchi-Ariga SM. Identification of heterochromatin protein 1 (HP1) as a phosphorylation target by Pim-1 kinase and the effect of phosphorylation on the transcriptional repression function of HP1(1). FEBS Lett 2000;467:17–21.

17. Mochizuki T, Kitanaka C, Noguchi K, Muramatsu T, Asai A, Kuchino Y. Physical and functional interactions between Pim-1 kinase and Cdc25A phosphatase. Implications for the Pim-1-mediated activation of the c-Myc signaling pathway. J Biol Chem 1999;274:18659–66.

18. Chen XP, Losman JA, Cowan S, et al. Pim serine/threonine kinases regulate the stability of Socs-1 protein. Proc Natl Acad Sci U S A 2002;99:2175–80.

19. Fingar DC, Salama S, Tsou C, Harlow E, Blenis J. Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. Genes Dev 2002;16:1472–87.

20. Grunwald V, DeGraffenried L, Russel D, Friedrichs WE, Ray RB, Hidalgo M. Inhibitors of mTOR reverse doxorubicin resistance conferred by PTEN status in prostate cancer cells. Cancer Res 2002;62:6141–5.

21. Gingras AC, Gygi SP, Raught B, et al. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. Genes Dev 1999;13:1422–37.

22. Gingras AC, Raught B, Gygi SP, et al. Hierarchical phosphorylation of the translation inhibitor 4E-BP1. Genes Dev 2001;15:2852–64.

23. Wang X, Li W, Parra JL, Beugnet A, Proud CG. The C terminus of initiation factor 4E-binding protein 1 contains multiple regulatory features that influence its function and phosphorylation. Mol Cell Biol 2003;23:1546–57.

24. Avdulov S, Li S, Michalek V, et al. Activation of translation complex eIF4F is essential for the genesis and maintenance of the malignant phenotype in human mammary epithelial cells. Cancer Cell 2004;5:553–63.

25. Herbert TP, Tee AR, Proud CG. The extracellular signal-regulated kinase pathway regulates the phosphorylation of 4E-BP1 at multiple sites. J Biol Chem 2002;277:11591–6.

26. Gingras AC, Kennedy SG, O'Leary MA, Sonenberg N, Hay N. 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. Genes Dev 1998;12:502–13.

27. Ionov Y, Le X, Tunquist BJ, et al. Pim-1 protein kinase is nuclear in Burkitt's lymphoma: nuclear localization is necessary for its biologic effects. Anticancer Res 2003;23:167–78.

28. Sarbassov DD, Ali SM, Kim DH, et al. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol 2004;14:1296–302.

29. Di Como CJ, Arndt KT. Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases. Genes Dev 1996;10:1904–16.

30. Reinhard C, Thomas G, Kozma SC. A single gene encodes two isoforms of the p70 S6 kinase: activation upon mitogenic stimulation. Proc Natl Acad Sci U S A 1992;89:4052–6.

31. Schalm SS, Tee AR, Blenis J. Characterization of a conserved C-terminal motif (RSPRR) in ribosomal protein S6 kinase 1 required for its mammalian target of rapamycin-dependent regulation. J Biol Chem 2005;280:11101–6.

32. De Benedetti A, Graff JR. eIF-4E expression and its role in malignancies and metastases. Oncogene 2004;23:3189–99.

33. Jefferies HB, Reinhard C, Kozma SC, Thomas G. Rapamycin selectively represses translation of the "polypyrimidine tract" mRNA family. Proc Natl Acad Sci U S A 1994;91:4441–5.

34. Sears R, Nuckolls F, Haura E, Taya Y, Tamai K, Nevins JR. Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. Genes Dev 2000;14:2501–14.

35. Welcker M, Orian A, Jin J, et al. The Fbw7 tumor suppressor regulates glycogen synthase kinase 3 phosphorylation-dependent c-Myc protein degradation. Proc Natl Acad Sci U S A 2004;101:9085–90.

36. Yeh E, Cunningham M, Arnold H, et al. A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. Nat Cell Biol 2004;6:308–18.

37. Raught B, Gingras AC. eIF4E activity is regulated at multiple levels. Int J Biochem Cell Biol 1999;31:43–57.

38. Tan A, Bitterman P, Sonenberg N, Peterson M, Polunovsky V. Inhibition of Myc-dependent apoptosis by eukaryotic translation initiation factor 4E requires cyclin D1. Oncogene 2000;19:1437–47.

39. Trumpp A, Refaeli Y, Oskarsson T, et al. c-Myc regulates mammalian body size by controlling cell number but not cell size. Nature 2001;414:768–73.

40. Hammerman PS, Fox CJ, Cinalli RM, et al. Lymphocyte transformation by Pim-2 is dependent on nuclear factor-κB activation. Cancer Res 2004;64:8341–8.

41. Duvel K, Santhanam A, Garrett S, Schneper L, Broach JR. Multiple roles of Tap42 in mediating rapamycin-induced transcriptional changes in yeast. Mol Cell 2003;11:1467–78.

42. Losman JA, Chen XP, Vuong BQ, Fay S, Rothman PB. Protein phosphatase 2A regulates the stability of Pim protein kinases. J Biol Chem 2003;278:4800–5.

43. Chen W, Possemato R, Campbell KT, Plattner CA, Pallas DC, Hahn WC. Identification of specific PP2A complexes involved in human cell transformation. Cancer Cell 2004;5:127–36.

44. Thyrell L, Hjortsberg L, Arulampalam V, et al. Interferon α-induced apoptosis in tumor cells is mediated through the phosphoinositide 3-kinase/mammalian target of rapamycin signaling pathway. J Biol Chem 2004;279:24152–62.

45. Dhawan P, Singh AB, Ellis DL, Richmond A. Constitutive activation of Akt/protein kinase B in melanoma leads to up-regulation of nuclear factor-κB and tumor progression. Cancer Res 2002;62:7335–42.

46. Zhang HM, Rao JN, Guo X, et al. Akt kinase activation blocks apoptosis in intestinal epithelial cells by inhibiting caspase-3 after polyamine depletion. J Biol Chem 2004;279:22539–47.

47. El-Hashemite N, Walker V, Zhang H, Kwiatkowski DJ. Loss of Tsc1 or Tsc2 induces vascular endothelial growth factor production through mammalian target of rapamycin. Cancer Res 2003;63:5173–7.

48. Inoki K, Li Y, Zhu T, Wu J, Guan KL. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol 2002;4:648–57.

49. Nave BT, Ouwens M, Withers DJ, Alessi DR, Shepherd PR. Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. Biochem J 1999;344 Pt 2:427–31.

50. Palaty CK, Clark-Lewis I, Leung D, Pelech SL. Phosphorylation site substrate specificity determinants for the Pim-1 protooncogene-encoded protein kinase. Biochem Cell Biol 1997;75:153–62.

51. Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. Genes Dev 1999;13:2905–27.

52. Mills GB, Lu Y, Kohn EC. Linking molecular therapeutics to molecular diagnostics: inhibition of the FRAP/RAFT/TOR component of the PI3K pathway preferentially blocks PTEN mutant cells *in vitro* and *in vivo*. Proc Natl Acad Sci U S A 2001;98:10031–3.

53. Neshat MS, Mellinghoff IK, Tran C, et al. Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. Proc Natl Acad Sci U S A 2001;98:10314–9.

54. Panwalkar A, Verstovsek S, Giles FJ. Mammalian target of rapamycin inhibition as therapy for hematologic malignancies. Cancer 2004;100:657–66.

55. Shi Y, Gera J, Hu L, et al. Enhanced sensitivity of multiple myeloma cells containing PTEN mutations to CCI-779. Cancer Res 2002;62:5027–34.

56. Vlietstra RJ, van Alewijk DC, Hermans KG, van Steenbrugge GJ, Trapman J. Frequent inactivation of PTEN in prostate cancer cell lines and xenografts. Cancer Res 1998;58:2720–3.

NEGATIVE REGULATION OF PIM-1 PROTEIN KINASE LEVELS BY THE B56β SUBUNIT OF PP2A [*]

Jian Ma[‡], Hugh K. Arnold[§], Michael B. Lilly[¶], Rosalie C. Sears[§], and Andrew S. Kraft[‡1]
From the ‡Hollings Cancer Center at the Medical University of South Carolina, Charleston, SC 29425, § Department of Molecular and Medical Genetics, Oregon Health & Sciences University, Portland, Oregon 97239, ¶ Chao Family Comprehensive Cancer Center, University of California, Irvine, Irvine, CA. 92868

Running Title: Regulation of Pim-1 by PP2A through B56β subunit

The Pim protein kinases are a group of serine threonine protein kinases that regulate important cellular signaling pathways including TOR and NF-κB, and enhance the ability of c-Myc protein to induce lymphomas. We demonstrate that a cascade of events control the cellular levels of Pim-1 protein kinase. We find that Pin1, a propyl isomerase, is capable of binding Pim-1 and leads to a decrease in the level of this protein. Additionally, we show that overexpression of PP2A catalytic subunit (PP2Ac) decreases the activity, phosphorylation, and the protein level of Pim-1. This effect is reversed by the application of okadaic acid, an inhibitor of PP2A, and is blocked by small T antigen that is known to dissociate PP2A subunits. Pim-1 does not bind to the A or C subunits of PP2A, but can coimmunprecipitate with the B56β subunit, but not B56α, γ, δ, ε or B55α. The binding of the B56β subunit to Pim-1 decreases its interaction with the A subunit. Using shRNA targeted at B56β, we demonstrate that decreasing the levels of this protein increases the half-life of Pim-1 from 0.7 to 2.8 hrs. This shRNA also decreases the level of ubiquitinylation of Pim-1. Based on these observations, we hypothesize that phosphorylated Pim-1 binds Pin1 allowing the interaction of PP2A through the B56β subunit. Dephosphorylation of Pim-1 then allows for ubiquitinylation and protein degradation of Pim-1.

The Pim family of serine/threonine protein kinases were initially identified as a target for proviral activation by Moloney murine leukemia virus (1,2). The Pim proteins have been implicated in the control of tumorigenesis, the cell cycle, and apoptosis (3-6). In animal models, Pim protein kinase have been shown to enhance development of lymphoma and prostate cancers, to be induced by c-Myc and Akt, and to be overexpressed in hepatocellular cancer (1,2,7,8). In humans, the protein levels of Pim have been shown to be elevated in lymphomas (9), leukemias (10), and prostate cancer (11,12). It is thought that Pim inhibits apoptosis by phosphorylating the BAD protein on S112, enhancing the binding and sequestering by 14-3-3 proteins, and enhancing the activity of Bcl-2 (13-15). Alternatively, Pim has been shown to regulate NF-κB activity and thus downstream proteins that play a role in apoptosis, i.e. Bax (16). The suggestion that Pim plays a role in the cell cycle comes from experiments that demonstrate that Pim protein kinases phosphorylate and regulate important cell cycle proteins including Cdc25A (17), p21waf (18), NuMA (19), C-TAK1 (20), and Cdc25C (20). Although overexpression of Pim appears to shorten the cell cycle (21), the exact biochemical mechanism by which Pim regulates the cell cycle has not been defined.

Because overexpression of Pim may regulate cancer growth or progression, the mechanism by which the levels of Pim proteins are controlled is important. Both Pim-1 and -2 proteins levels are elevated transcriptionally by the application of IL-3, GM-CSF, IL-7, and other cytokines to cells (5). This transcriptional induction appears to be regulated by the JAK/STAT/SOCS family of proteins (22). Additionally, there may be a feedback where Pim proteins phosphorylate SOCS-1 increasing its half-life and blocking growth factor signals (23,24). Mitogen stimulation can regulate the stability of Pim protein kinase mRNA (25). Once increased, Pim protein kinases have a relatively short half-life (about 10 min). This suggests that the degradation of Pim proteins may play a crucial role in regulating the half-life of this protein. Both HSP70 and 90 have been implicated in the stabilization of this protein (26,27), and more

recently the phosphatase PP2A (28) and ubiquitinylation (26) has been suggested to play a role.

PP2A is a heterotrimeric protein with an A subunit that serves as a scaffold that binds the catalytic subunit (C) and the regulatory subunit (B). These then interact directly with varied proteins. The A and C subunits each exist in two isoforms, while there are 25 different B subunits which have been identified falling into four families, including B, B', B'', and B'''. Combinations of these subunits lead to the possibility of 75-100 different protein phosphatases that account for 30-50% of cellular phosphatase activity. PP2A has been shown to regulate varied cellular properties including proliferation, growth, differentiation and apoptosis (29-32).

A critical role for PP2A in transformation has been suggested by the observation that SV-40 small T (ST) antigen, which is necessary for the transformation of human cells along with SV-40 large T, hTERT, and H-Ras, dissociates PP2A. Loss of the ability of ST to inhibit PP2A blocks its transforming ability. Elevation of the PP2A B56γ_3 subunit reverses this effect and blocks transformation, suggesting that this phosphatase acts as a tumor suppressor (33). In addition, PP2A binds the adenomatous polyposis coli (APC) tumor suppressor (34) and cyclin G (35). A further suggestion for the important role of PP2A in human neoplasia comes from the observation that human cancers, e.g. breast cancer, harbor mutations in the A subunit that prevent binding of either the B or C subunit and thus inhibit PP2A activity (36).

PP2A has also been shown to play a significant role in controlling apoptosis (37). PP2A C subunit knockout mice die in early embryogenesis and in all organisms examined the C subunit is necessary for cell survival (38). In rat PC6-3 cells suppression of the Aα subunit induced both apoptotic and nonapopototic cell death (39,40). Further control of apoptosis may come in the form of regulation of pro- and anti-apoptotic molecules. PP2A dephosphorylates and alters the activity of the BH3 proteins BAD, and causes the dephosphorylation of Bcl-2 in a ceramide-dependent fashion (41,42). Many additional proteins implicated in cell survival or transformation have been shown to be regulated by PP2A, including MAPK, Wnt, phosphatidylinositol 3-kinase, NF-kB, PKC, and calcium/calmodulin dependent kinase (29,31,43).

The ability of PP2A to modulate the dephosphorylation of c-Myc, and thus control its degradation, depends on the binding and regulation of c-Myc by the Pin1 propyl isomerase. Isomerization of proline residues by this protein has been shown to promote dephosphorylation of the molecule by PP2A (44,45). Pin1 appears to play a significant role in cellular transformation. Pin1 binds Ser/Thr-Pro motifs through its WW motif and binds to a diverse set of substrates, including Cdc25 and Tau (46,47). Pin1 regulates the activity of p53 (48). In Pin1 (-/-) fibroblasts, p53 activates a diminished set of substrates. Overexpression of Pin1 confers transformed properties on mammary epithelial cells and inhibition of this molecule suppresses Neu/Ras transformed phenotypes in these cells (49).

Because of the importance of Pim-1 cell levels to its ability to potentially control cell apoptosis and enhance transformation, we have studied the biochemical control of Pim-1 levels in some detail. We find that PP2A regulates the level of Pim-1 and this effect is blocked by ST and okadaic acid, two inhibitors of PP2A. Although there are multiple PP2A B subunits, PP2A B56β subunit appears to bind strongly to Pim-1. The B56β subunit is found in a complex with Pin1, and overexpression of Pin1 decreases the level of Pim-1. The B56β protein regulates the level of Pim-1 ubiquitinylation and thus controls the level of this protein. These data suggest a complex regulation of Pim-1 protein which could be targeted during transformation.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Anti-Flag M2 Agarose, protease inhibitor cocktail, N-ethylmaleimide, okadaic acid, and cycloheximide were purchased from Sigma (St. Louis, MO). The protein A/G agarose was obtained from Calbiochem (La Jolla, CA), anti-HA antibody from Abcam (Cambridge, MA) and the anti-GAPDH antibody from Chemicon (Temecula, CA). The anti-His antibody was purchased from Qiagen (Valencia, CA), and both anti-Pim-1 (12H8) and anti-Pim-2 (1D12) antibodies were

obtained from Santa Cruz Biotechnology (Santa Cruz, CA). [^{32}P] orthophosphate and [γ-^{32}P] ATP were obtained from Perkin Elmer (Wellesley, MA), recombinant PP2A and histone H1 from Upstate (Chicago, IL), and mouse IL-3 from R&D Systems (Minneapolis, MN). Bortezomib (Velcade, PS-341) was a gift of Millenium, Inc. (Cambridge, MA).

Cell Culture and Transfetion—Human embryonic kidney (HEK) 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin at 37°C in 5% CO_2. Cells were plated to achieve 50 to 70% confluence 24 h prior to transfection. Transfections were performed using Effectene (Qiagen, Valencia, CA) according to the instructions of the manufacturer. Transfected cells were maintained in DMEM supplemented with 10% FBS, except in shRNA experiments, in which they were maintained in 2% or 0.2% FBS for the indicated time periods. The murine hematopoietic cell line, BaF3, was cultured in RPMI 1640 medium supplemented with 10% FBS and 1 ng/ml mouse IL-3.

Plasmids—Constructs encoding pD30-PP2A-FLAG-A, pD30-PP2A-HA-C, small hairpin RNA (shRNA) B56β have been described previously (44). Expression vectors for pCEP4HA-B56α, -β, -γ, -δ, and ε, were a generous gift from David Virshup (Huntsman Cancer Institute, University of Utah) (50). pCEP-small-T-antigen expression vector was kindly provided by William C. Hahn (Dana-Farber Cancer Institute, Harvard Medical School) (33). pcDNA3/Flag-Pim-1(murine), pcDNA3/Flag-Pim-2(murine), pcDNA3/HA-Pim-1(murine) have been described previously (21). pcDNA3/HA-Pin1 was a gift from Ilan R. Kirsch (Research Oncology, Amgen, 1201 Amgen Court West, AW1-J4144, Seattle, WA) (51). HA-ubiquitin was a gift from Mathias Treier (European Molecular Biology Laboratory, Heidelberg, Germany) (52). shRNA Pin1 (TRCN0000001034) was purchased from Sigma.

Western Blotting— Cell extracts were prepared by lysing cells in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1% NP-40, 1 mM β-glycerolphosphate, 1 mM NaF, 1 mM Na_3VO_4, 1mM DTT, and protease inhibitor cocktail (Sigma). The lysates were incubated on ice for 15 minutes followed by centrifugation for 10 minutes

at 4°C. The supernatant was subjected to protein concentration detection by using the DC (detergent compatible) Protein assay kit (Bio-Rad, Hercules, CA). Then, the supernatant was combined with 4x Laemmli's SDS-sample buffer and the mixture was heated at 100°C for 5 minutes. The cell extracts (50-100 μg) were resolved by SDS-PAGE and then transferred to Immobilon-P membrane filters (Millipore, Bedford, MA). Membranes were blocked in 5% BSA (or non-fat milk) in TBS buffer. Primary antibodies were in 5% BSA (or non-fat milk), 0.1% Tween20, TBS and were detected with HRP-conjugated secondary antibodies using the ECL reagents from Amersham (Piscataway, NJ).

Immunoprecipitations—Cleared cell lysates were incubated with either anti-Flag M2 Agarose for 3 h or HA antibody for 3 h followed by the addition of protein A/G agarose for 1 h. Immunoprecipitates were washed three times with cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.1% SDS, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, protease inhibitor cocktail).

Detection of Ubiquitinylated Pim-1—The method has been described previously (45). Briefly, HEK-293T cells were contransfected with 1μg CMV-HA-ubiquitin, 1μg pcDNA3/Flag-Pim-1, and 4 μg either shRNA-empty or shRNA-B56β plasmids for 48 h. Cells were washed once with cold PBS and lysed with RIPA buffer containing standard protease and phosphatase inhibitors, with the addition of the de-ubiquitinase inhibitor 5 mM N-ethylmaleimide (Sigma) at a cell-to-volume ratio of 1x10^6 cells ml^{-1}. Ubiquitinylated proteins were immunoprecipitated from cell lysates with an anti-Flag M2 agarose for 3 hours. Immunoprecipitates were washed once with low-stringency buffer containing PBS, 0.1% NP40 and all inhibitors contained in the RIPA buffer. Immunoprecipitated proteins were then separated by SDS-PAGE, blotted to Immobilon-P membranes and detected with the indicated antibodies.

In vitro Kinase Assay— HEK-293T Cells were transfected with Flag-Pim-1, and PP2Ac or empty vector, as indicated. For the Pim-1 immunoprecipitation experiments, 1 mg of total cellular lysate in a total volume of 0.5 ml of lysis buffer was incubated with anti-Flag M2 Agarose and mixed at 4°C for 3 h. The beads were

collected by centrifugation, unbound protein removed by multiple washes in PBS and then resuspended in kinase reaction buffer (10 mM MOPS, pH7.4, 100 μM ATP, 15 mM MgCl$_2$, 1 mM Na$_3$VO$_4$, 1 mM NaF, 1mM DTT, and protease inhibitor cocktail). In each reaction system (30 μl), 3 μg of histone H1 protein (Upstate) was used as substrate, and 10 μCi of [γ-^{32}P] ATP were then added and incubated at 30°C for 30 min with agitation. Labeled protein was then resolved on a 16% SDS-PAGE, the gel dried and then exposed to film.

Dephosphorylation Assay —To examine dephosphorylation of Pim-1 *in vivo*, HEK-293T cells were transfected with Flag-Pim-1, and either PP2Ac or empty vector, as indicated for 36 h, then washed once and incubated with phosphate-free media containing 10% phosphate-free FBS(Invitrogen, Carlsbad, CA) for 1 h. Then, cells were incubated 4 h in medium containing 50 μCi/ml [^{32}P] orthophosphate. For immunoprecipitation, anti-Flag M2 Agarose was added to each cell lysate sample, followed by 3 h incubation on a rocker at 4°C. Immunoprecipitates were then washed three times in lysis buffer, followed by heating at 100°C for 5 min in SDS sample buffer prior to SDS-PAGE.

To study the *in vitro* dephosphorylation of this enzyme, the cells were washed once and then incubated with phosphate-free media containing 10% phosphate-free FBS for 1 h. HEK-293T cells transfected with Flag-Pim-1 were incubated 4 h in medium containing 50 μCi/ml [^{32}P] orthophosphate. For immunoprecipitation, anti-Flag M2 Agarose was added to each cell lysate sample, followed by 3 h incubation on a rocker at 4°C. Immunoprecipitates were washed once in phosphatase buffer (50 mM HEPES, pH7.5, 0.5% bovine serum albumin, and 1 mM DTT), and then resuspended in 40 μl of the same buffer. Recombinant PP2A (A/C dimmer) (Upstate, Chicago, IL) was then added and incubated at 30°C for 30 min with agitation. The reaction was terminated by washing twice in 50 mmol/L HEPES, pH8.0, followed by heating at 100°C for 5 min in SDS sample buffer in preparation for SDS-PAGE.

Pim-1 Half-life—First, 100-mm dishes of HEK-293T cells were transfected with varied plasmids for 24 h. Then each transfection mixture was split into six 60-mm dishes, maintained for 24

h in DMEM supplemented with 10% FBS, and then starved in DMEM supplemented with 0.2% FBS for 48 h. Cells were treated with 100 μg/ml cycloheximide 5 min prior to starting the indicated time course, and cells were collected at the indicated points.

Cell Proliferation detection by using a modified MTT assay (MTS)—BaF3 cells were transfected with 2 μg shRNA-empty vector or shRNA-B56β for 48 h. Cell number of each transfection mixture was counted and seeded in 96-well dishes at 1,250 cells/well. After 48 hours, the total cell number was determined by a modified 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS); Promega, Madison, WI). Metabolically active cells were quantified by the bioreduction of the MTS tetrazolium compound by recording absorbance at 490 nm using a microplate reader. Results were calculated from the average of three separate experiments and are reported as the percentage of treated cells relative to the cells in control wells (% Control).

RESULTS

Increased PP2A Activity Negatively Regulates Pim-1 Protein Levels—To further characterize the role of PP2A in regulating Pim-1 protein levels, we examined the effects of increasing cellular PP2A activity on Pim-1 protein levels. We coexpressed the cDNAs for Pim-1 or Pim-2 with increasing amounts of PP2A catalytic (PP2A-HA-C) subunit (53,54). As shown in Fig. 1A, increasing amounts of PP2A-HA-C reduced both Pim-1 and Pim-2 protein levels. As a negative control, the PP2A-HA-C did not reduce the protein levels of RUNX1. Okadaic acid is a strong inhibitor to PP2A with lesser activity against PP1 (55). Treatment of Pim-1 transfected 293T cells with okadaic acid dramatically increased the level of Pim-1(Fig.1B). This data demonstrates that the level of both Pim kinases is negatively regulated by the activity of PP2A.

PP2A Dephosphorylates Pim-1 both in vivo and in vitro, and Decreases Pim-1 Kinase Activity—PP2A is one of the major serine/threonine phosphatases implicated in the regulation of multiple cellular processes. PP2A controls the activity of at least 50 different protein

kinases (31,43). The Pim-1 kinases are known to be phosphorylated on multiple serine and threonines sites (56). To determine whether PP2A can directly dephosphorylate Pim-1 kinase, both *in vivo* and *in vitro* dephosphorylation assays were performed. 293T cells were labeled with [^{32}P] orthophosphate after transfection with cDNAs encoding PP2Ac and Pim-1 and the amount of phosphorylated Pim measured by immunoprecipitation followed by SDS-PAGE. The *in vivo* results demonstrate that cotransfection of Pim-1 and PP2Ac can decrease the phosphorylation level of Pim-1 protein when compared to the transfection of Pim-1 alone (Fig.2A). Because it is possible that the effect of PP2Ac is indirect, Pim-1 was labeled with [^{32}P] *in vivo*, immunoprecipitated and then incubated *in vitro* with recombinant PP2A (A/C dimmer). This assay demonstrated that PP2Ac can directly dephosphorylate Pim-1 protein (Fig.2B). To examine whether the phosphorylation of Pim-1 regulates its activity, Pim-1 immunoprecipitates from 293T cells were subjected to a kinase assay using histone H1 as a substrate. Fig.2C demonstrates that cotransfection with Pim-1 and PP2Ac can decrease the kinase activity of Pim-1 compared to the transfection of Pim-1 alone. Taken together, these results demonstrate that PP2Ac can dephosphorylate Pim-1 and decrease Pim-1 kinase activity.

PP2A B56β Associates with Pim-1 in vivo —In vivo, PP2A is a holoenzyme consisting of an A, B and C subunit. The A subunit functions as a scaffold while the B subunit specifies substrate recognition. SV40 small T antigen (ST) can compete with the PP2A B55α subunit and some B56 subunits for binding to the PP2A-A and -C subunits and thus inhibit PP2A activity (32,33). To examine whether small T could inhibit the ability of PP2A to decrease Pim-1 levels, the cDNA encoding small T protein was cotransfected with Pim-1 and PP2Ac (Fig.3A). The result showing that small T can indeed block the destruction of Pim-1 suggests that PP2Ac must associate with the A and B subunits of the enzyme to decrease Pim-1 levels.

To evaluate whether Pim-1 directly interacts with one of these subunits we examined the ability of this enzyme to coimmunoprecipitate with A, B or C subunits. We did not find any interaction with either A, C or B55α (data not shown). Next we focused on the role of B56 family in the regulation of Pim-1 by PP2A. Regulatory B subunits usually associate with the substrate and facilitate the C subunit to dephosphorylate the target protein. This prompted us to determine which B56 subunit can interact with Pim-1 kinase. Cells were cotransfected with pcDNA3/Flag-Pim-1 and either HA-tagged B56α,-β,-γ,-δ,-ε subunit or empty control. As shown in Fig.3B, Pim-1 coimmunoprecipitated with anti-HA antibody only in the presence of the B56β subunit (Fig.3B upper panel). Alternatively, when Pim-1 is immunoprecipitated with anti-Flag beads, only the B56β subunit is co-immunoprecipitated with Pim-1 (Fig.3C upper panel). These coimmunoprecipitation results reveal that among the B56 family members, only B56β can associate with Pim-1 *in vivo*. This suggests that the PP2A holoenzyme associate with Pim-1 through the B56β regulatory subunit.

To further study the role of B56β we have used a specific shRNA to decease the expression of the B56β (44). We demonstrated in Fig.4A that the shRNA B56β specifically reduced expression of B56β but not other B56 subunits. The constransfection of Pim-1 with increasing amount of either shRNA B56β or vector control demonstrates that increasing the knockdown of B56β protein, further increased Pim-1 protein levels. The control shRNA vector did not affect the Pim-1 expression (Fig.4B). To determine whether PP2A-B56β can regulate the stability of Pim-1 protein, we transfected Pim-1 and then incubated cells with cycloheximide to block further protein synthesis. As shown in Fig.4C, knockdown of B56β significantly decreases the rate of Pim-1 protein degradation and increases the half life of Pim-1 from 0.7 h to 2.8 h. Together these results suggest that B56β is likely the major regulatory B subunit responsible for targeting PP2A activity to Pim-1.

B56β Affects the Level of Pim-1 Ubiquitinylation—In the case of the c-Myc protein, dephosphorylation of the protein by PP2A leads to ubiquitinylation and protein degradation (45). To evaluate whether B56β activity is important for Pim-1 ubiquitinylation, we examined the effect of B56β knockdown on Pim-1 ubiquitinylation levels. To allow us to compare levels of protein ubiquitinylation, 293T cells transfected with Pim-1 were treated with the proteasome inhibitor

Bortezomib (57) for 6 h to prevent the degradation of Pim-1 protein. Fig.4D shows that ubiquitinylation of Pim-1 is decreased upon B56β knockdown when compared to the control group, although the total levels of Pim-1 was increased upon shRNA-B56β treatment. These results revealed that the increase of Pim-1 protein levels upon knockdown of B56β is due in part to decreased Pim-1 protein ubiquitinylation.

The Pin1 Prolyl Isomerase Associates with Pim-1 in vivo and Facilitates Pim-1 Degradation —As reported, PP2A is a conformation-sensitive protein phosphatase, preferring the *trans* configuration of proline residue adjacent to the phosphor-serine or -threonine in substrates (46). The prolyl isomerase Pin1 can catalyse the isomerization of proline residues in phospho-proteins to promote their dephosphorylation by PP2A (47). We therefore evaluated whether Pim-1 could bind Pin1 and whether the later could affect the levels of Pim-1. As shown in Fig.5A, Pim-1 can coimmunoprecipitate Pin1 when both are transfected into 293T cells, Interestingly Pim-1 can bind and coimmunoprecipitate Pin1 and B56β simultaneously suggesting that they form a complex *in vivo*. Fig.5B (upper panel) demonstrates that by using the shRNA we can decrease the Pin1 expression approximately 70%. We next examined whether Pin1 expression affected the level of Pim-1 protein by using shRNA targeting Pin1. Knock-down of Pin1 by using shRNA led to a 2-3 fold increase in Pim-1 protein levels. These results are consistent with the model that phospho-Pim binds Pin1 leading to a configuration change that encourages the binding of PP2A, dephosphorylation of the protein leading to ubiquitinylation and degradation.

B56β Associates with the Hinge Region of Pim-1 and Pim-1 Associates with ASBD of B56β— To identify the binding site of B56β on the Pim-1 protein, we prepared deletion mutants of Pim-1, as indicated in Fig.6A. The HA-tagged B56β was expressed in 293T cells along with the Flag-tagged full length- or deleted Pim-1 protein and the Pim-1 proteins immunoprecipitated. Fig 6B demonstrates B56β coimmunprecipitates with full length (1-313), ΔN (69-313), ΔC (1-250), and ΔC (1-177) Pim-1, but not with ΔN (140-313). These results indicate that the amino acid residues 70-139 of Pim-1 are responsible for the interaction to B56β. This sequence contains the hinge region (121-126)

of the Pim-1 protein kinase which includes the ATP binding pocket (58). Next, we analyzed which region of the B56β bound to Pim-1. We prepared deletion mutants of B56β, as indicated in Fig.6C. Coimmunoprecipitation experiments demonstrated that full length (1-497), ΔN (76-497), ΔN (138-497), ΔC (1-467), and ΔC (1-390) B56β all formed a complex with Pim-1 (Fig.6D). This result suggested that the overlapping area between these clones which contains the regions of 206-389 that interacts with the A subunit (ASBD, PP2A-A subunit binding domain 1 and 2) (59) could be the domain that interacts directly with Pim-1. As seen in Fig. 6E, the ASBD domain can bind directly to Pim-1. Since A subunit and Pim-1 both bind to the B56β, we then examined whether there is a competition between these two proteins for binding to B56β. As shown in Fig.6F, in immunoprecipitates of the A subunit we found less B56β coimmunoprecipitating if Pim-1 was included in the transfection (compare middle lane with Pim-1 to right lane without). This result suggests that Pim-1 binding to the B56β inhibits interaction of the A and B subunit.

Endogenous Pim-1 Protein Levels are Decreased by PP2A Overexpression —To examine whether the PP2A-B56β regulates not only transfected Pim-1 levels but the endogenous Pim-1 protein levels, we have used the murine hematopoietic cell line BaF3, which contains a measurable level of endogenous Pim-1 protein (60). Fig.7A shows that increasing cellular PP2A activity via transfection can decrease endogenous 33 and 44 kDa Pim-1 protein levels. In contrast, Fig.7B and C show that the addition of okadaic acid or shRNA B56β can increase the endogenous Pim-1 protein levels while the control shRNA had no effect. In hematopoietic cells, Pim-1 is involved in cytokine-dependent signaling via its ability to regulate activities of transcription factors, enhance hematopoietic cell survival, and participate in the regulation of the cell cycle (5). We used a modified MTT assay to detect the effect of knockdown of B56β on BaF3 cells proliferation. As shown in Fig.7D, knockdown of B56β can increase the cell proliferation by 37% when compared to

control shRNA empty vector transfected cells. This result may be due to the increase of Pim-1 protein levels induced by the knockdown of B56β protein, although other targets for this subunit cannot be ruled out.

DISCUSSION

We demonstrate in 293T cells transfected with Pim-1 and Pim-2 cDNAs, as well as in Ba/F3 cells expressing endogenous levels of Pim-1, that the overexpression of the PP2Ac subunit decreases the level of Pim proteins. These results extend the work of Losman JA *et al.* (28) who have demonstrated similar findings for Pim-1 and -3. This effect of PP2Ac is reversed by both okadaic acid, a known inhibitor of the PP2A complex and additionally the ST protein of SV-40 (32). ST is thought to dissociate the PP2A complex by binding to the A subunit and displaced a B subunit from the A subunit (61) leading to cellular transformation. This result suggests that the entire enzyme, not only the C subunit is needed to decrease Pim-1 levels (see below). By labeling cells with ^{32}P-orthophosphate in the presence of a proteasome inhibitor, we have been able to demonstrate that PP2Ac actually dephosphorylates the Pim-1 protein. *In vitro* recombinant PP2A (A/C dimmer) is able to accomplish the same effect on Pim-1, replicating the observation made previously with Pim-3 (28). This decrease in phosphorylation of Pim-1 is associated with a decreased ability of Pim-1 to phosphorylate an artificial substrate, suggesting that phosphorylation of Pim-1 controls its activity. Crystallography data has suggested that that Pim-1 autophosphorylates and is active without an additional protein kinase cascade controlling its ability to modify substrates (56). It is interesting that Pim-1 has been shown to transform cells in concert with both c-Myc (8,62,63) or Akt (3), and that the levels of both of these proteins are regulated by PP2A (64-66).

PP2A is targeted to specific substrates through its B subunit. The ST protein targets the PP2A-B56γ₃ protein while c-Myc interacts with the B56α subunit. The activity of ceramide on Bcl-2 is thought to be mediated through the B56α protein (42). We demonstrate by co-immunoprecipitation, even in the presence of 2% NP-40, that the B56β subunit of PP2A interacts strongly with Pim-1. The suggestion that B56β is important is further strengthened by experiments in which shRNA B56β into either 293T or BaF3 cells and the level of either transfected or endogenous Pim-1 protein is elevated. Cutbacks in the B56β subunit demonstrate that a fragment of the protein from 206 to 379 binds to the Pim-1 protein. This is a region that is approximately 70% conserved among B subunits; a similar region of this B subunit interacts with the A subunit of PP2A (59). By transfecting 293T cells with the B56β subunit, the A subunit and Pim-1, we demonstrate that Pim-1 can actually compete with the A subunit for B56β subunit binding. This data suggests a model whereby the PP2A holoenzyme binds to Pim-1 and dephosphorylates the protein. The Pim-1 protein, through interaction with the B subunit, inhibits the B subunit binding to A subunit, then decreases the activity of PP2A. We have demonstrated an effect of Pim-1 on PP2A activity in prostate cancer cells (21). One could hypothesize that overexpressed Pim-1 might enhance transformation by decreasing the activity of the PP2A enzyme.

Shay *et al.* (26) have demonstrated that Pim-1 is conjugated to ubiquitin and that inhibition of the proteasomal degradation increases its half-life. We show that decreasing the level of B56β subunit by shRNA decreases the ubiquitinylation of Pim-1 and increases the half-life of this protein from 0.7 to 2.8 h. These results suggest that the degradation of Pim-1 could be regulated in a similar fashion to c-Myc and other proteins(45). In this case, PP2A dephosphorylation leaves a partially phosphorylated protein, for example c-Myc with T58 phosphorylated, that is an excellent target for a specific E3 ligase. We have mutated Ser189 of Pim-1, that is a potential autophosphorylation site of Pim-1(67) but did not detect any change in half-life, suggesting that further mapping of the Pim-1 phosphorylation sites will be important to understanding the regulation of the levels of this protein.

Coimmunoprecipitation experiments demonstrate that the B56β subunit can bind to Pim-1 in the region between amino acids 70-139. This region contains the hinge portion of the protein between the two lobes and the ATP binding pocket (58,68,69). Our results do not

exclude the possibility that B56β competes with ATP to interact with this portion of the protein. We demonstrate that Pin1 and B56β form a complex with Pim-1. Murine Pim-1 contains five potential SP docking sites for Pin1. In murine Pim-1, two of these SP sites are located close together S55 and S61. The presence of two closely phosphorylated SP motifs, for example in c-Myc and RNA polymerase II, increases the binding affinity and catalytic efficacy of Pin1 towards these substrates (45,70). Further experiments will be necessary to map the phosphorylation sites on Pim-1, the potential binding sites of Pin-1, and the sites dephosphorylated by PP2A.

Transfection of the shRNA of B56β into BaF3 cells increases the levels of Pim-1 and enhances the growth of these cells as measured by the MTS assay. Previously we have shown in human prostate cancer cells that overexpression of Pim-1 shortens the cell cycle and increases the growth rate of these cells. It is possible that the increased levels of Pim-1 are inducing changes in the cell cycle leading to increased cell numbers. However, different mechanisms could be operative. B56β may bind to other substrates and regulate the cell cycle using a mechanism separate from Pim-1.

Our data suggests the possibility that Pim-1 levels are being regulated in a similar fashion to c-Myc (44,45). Pin1 binds to phosphorylated Pim-1 and enhances the ability of the PP2A holoenzyme to bind to the complex through the B56β protein. PP2A dephosphorylates Pim-1 protein, and decreases its activity. The partially phosphorylated protein is then ubiquitinylated and degraded by the proteasome. Additional experiments will be necessary to completely validate this model and examine whether in human neoplasms this mechanism is defective leading to increased levels of Pim protein.

REFERENCES

1. Cuypers, H. T., Selten, G., Quint, W., Zijlstra, M., Maandag, E. R., Boelens, W., van Wezenbeek, P., Melief, C., and Berns, A. (1984) *Cell* **37**(1), 141-150
2. Selten, G., Cuypers, H. T., and Berns, A. (1985) *Embo J* **4**(7), 1793-1798
3. Amaravadi, R., and Thompson, C. B. (2005) *J Clin Invest* **115**(10), 2618-2624
4. Bachmann, M., and Moroy, T. (2005) *Int J Biochem Cell Biol* **37**(4), 726-730
5. Wang, Z., Bhattacharya, N., Weaver, M., Petersen, K., Meyer, M., Gapter, L., and Magnuson, N. S. (2001) *J Vet Sci* **2**(3), 167-179
6. Mikkers, H., Nawijn, M., Allen, J., Brouwers, C., Verhoeven, E., Jonkers, J., and Berns, A. (2004) *Mol Cell Biol* **24**(13), 6104-6115
7. van Lohuizen, M., Verbeek, S., Krimpenfort, P., Domen, J., Saris, C., Radaszkiewicz, T., and Berns, A. (1989) *Cell* **56**(4), 673-682
8. Ellwood-Yen, K., Graeber, T. G., Wongvipat, J., Iruela-Arispe, M. L., Zhang, J., Matusik, R., Thomas, G. V., and Sawyers, C. L. (2003) *Cancer Cell* **4**(3), 223-238
9. Ionov, Y., Le, X., Tunquist, B. J., Sweetenham, J., Sachs, T., Ryder, J., Johnson, T., Lilly, M. B., and Kraft, A. S. (2003) *Anticancer Res* **23**(1A), 167-178
10. Adam, M., Pogacic, V., Bendit, M., Chappuis, R., Nawijn, M. C., Duyster, J., Fox, C. J., Thompson, C. B., Cools, J., and Schwaller, J. (2006) *Cancer Res* **66**(7), 3828-3835
11. Cibull, T. L., Jones, T. D., Li, L., Eble, J. N., Ann Baldridge, L., Malott, S. R., Luo, Y., and Cheng, L. (2006) *J Clin Pathol* **59**(3), 285-288

12. Xu, Y., Zhang, T., Tang, H., Zhang, S., Liu, M., Ren, D., and Niu, Y. (2005) *J Surg Oncol* **92**(4), 326-330
13. Kim, K. T., Levis, M., and Small, D. (2006) *Br J Haematol* (Epub ahead of print)
14. Macdonald, A., Campbell, D. G., Toth, R., McLauchlan, H., Hastie, C. J., and Arthur, J. S. (2006) *BMC Cell Biol* **7**, 1
15. Aho, T. L., Sandholm, J., Peltola, K. J., Mankonen, H. P., Lilly, M., and Koskinen, P. J. (2004) *FEBS Lett* **571**(1-3), 43-49
16. Hammerman, P. S., Fox, C. J., Cinalli, R. M., Xu, A., Wagner, J. D., Lindsten, T., and Thompson, C. B. (2004) *Cancer Res* **64**(22), 8341-8348
17. Mochizuki, T., Kitanaka, C., Noguchi, K., Muramatsu, T., Asai, A., and Kuchino, Y. (1999) *J Biol Chem* **274**(26), 18659-18666
18. Wang, Z., Bhattacharya, N., Mixter, P. F., Wei, W., Sedivy, J., and Magnuson, N. S. (2002) *Biochim Biophys Acta* **1593**(1), 45-55
19. Bhattacharya, N., Wang, Z., Davitt, C., McKenzie, I. F., Xing, P. X., and Magnuson, N. S. (2002) *Chromosoma* **111**(2), 80-95
20. Bachmann, M., Kosan, C., Xing, P. X., Montenarh, M., Hoffmann, I., and Moroy, T. (2006) *Int J Biochem Cell Biol* **38**(3), 430-443
21. Chen, W. W., Chan, D. C., Donald, C., Lilly, M. B., and Kraft, A. S. (2005) *Mol Cancer Res* **3**(8), 443-451
22. Stout, B. A., Bates, M. E., Liu, L. Y., Farrington, N. N., and Bertics, P. J. (2004) *J Immunol* **173**(10), 6409-6417
23. Peltola, K. J., Paukku, K., Aho, T. L., Ruuska, M., Silvennoinen, O., and Koskinen, P. J. (2004) *Blood* **103**(10), 3744-3750
24. Chen, X. P., Losman, J. A., Cowan, S., Donahue, E., Fay, S., Vuong, B. Q., Nawijn, M. C., Capece, D., Cohan, V. L., and Rothman, P. (2002) *Proc Natl Acad Sci U S A* **99**(4), 2175-2180
25. Fox, C. J., Hammerman, P. S., Cinalli, R. M., Master, S. R., Chodosh, L. A., and Thompson, C. B. (2003) *Genes Dev* **17**(15), 1841-1854
26. Shay, K. P., Wang, Z., Xing, P. X., McKenzie, I. F., and Magnuson, N. S. (2005) *Mol Cancer Res* **3**(3), 170-181
27. Mizuno, K., Shirogane, T., Shinohara, A., Iwamatsu, A., Hibi, M., and Hirano, T. (2001) *Biochem Biophys Res Commun* **281**(3), 663-669
28. Losman, J. A., Chen, X. P., Vuong, B. Q., Fay, S., and Rothman, P. B. (2003) *J Biol Chem* **278**(7), 4800-4805
29. Janssens, V., Goris, J., and Van Hoof, C. (2005) *Curr Opin Genet Dev* **15**(1), 34-41
30. Lechward, K., Awotunde, O. S., Swiatek, W., and Muszynska, G. (2001) *Acta Biochim Pol* **48**(4), 921-933
31. Janssens, V., and Goris, J. (2001) *Biochem J* **353**(Pt 3), 417-439
32. Arroyo, J. D., and Hahn, W. C. (2005) *Oncogene* **24**(52), 7746-7755
33. Chen, W., Possemato, R., Campbell, K. T., Plattner, C. A., Pallas, D. C., and Hahn, W. C. (2004) *Cancer Cell* **5**(2), 127-136
34. Seeling, J. M., Miller, J. R., Gil, R., Moon, R. T., White, R., and Virshup, D. M. (1999) *Science* **283**(5410), 2089-2091
35. Okamoto, K., Li, H., Jensen, M. R., Zhang, T., Taya, Y., Thorgeirsson, S. S., and Prives, C. (2002) *Mol Cell* **9**(4), 761-771
36. Ruediger, R., Pham, H. T., and Walter, G. (2001) *Oncogene* **20**(1), 10-15

37.   Van Hoof, C., and Goris, J. (2003) *Biochim Biophys Acta* **1640**(2-3), 97-104
38.   Gotz, J., Probst, A., Ehler, E., Hemmings, B., and Kues, W. (1998) *Proc Natl Acad Sci U S A* **95**(21), 12370-12375
39.   Strack, S., Cribbs, J. T., and Gomez, L. (2004) *J Biol Chem* **279**(46), 47732-47739
40.   Strack, S., Ruediger, R., Walter, G., Dagda, R. K., Barwacz, C. A., and Cribbs, J. T. (2002) *J Biol Chem* **277**(23), 20750-20755
41.   Chen, C. L., Lin, C. F., Chiang, C. W., Jan, M. S., and Lin, Y. S. (2006) *Mol Pharmacol* **70**(2), 510-517
42.   Ruvolo, P. P., Clark, W., Mumby, M., Gao, F., and May, W. S. (2002) *J Biol Chem* **277**(25), 22847-22852
43.   Millward, T. A., Zolnierowicz, S., and Hemmings, B. A. (1999) *Trends Biochem Sci* **24**(5), 186-191
44.   Arnold, H. K., and Sears, R. C. (2006) *Mol Cell Biol* **26**(7), 2832-2844
45.   Yeh, E., Cunningham, M., Arnold, H., Chasse, D., Monteith, T., Ivaldi, G., Hahn, W. C., Stukenberg, P. T., Shenolikar, S., Uchida, T., Counter, C. M., Nevins, J. R., Means, A. R., and Sears, R. (2004) *Nat Cell Biol* **6**(4), 308-318
46.   Zhou, X. Z., Kops, O., Werner, A., Lu, P. J., Shen, M., Stoller, G., Kullertz, G., Stark, M., Fischer, G., and Lu, K. P. (2000) *Mol Cell* **6**(4), 873-883
47.   Stukenberg, P. T., and Kirschner, M. W. (2001) *Mol Cell* **7**(5), 1071-1083
48.   Zacchi, P., Gostissa, M., Uchida, T., Salvagno, C., Avolio, F., Volinia, S., Ronai, Z., Blandino, G., Schneider, C., and Del Sal, G. (2002) *Nature* **419**(6909), 853-857
49.   Ryo, A., Liou, Y. C., Wulf, G., Nakamura, M., Lee, S. W., and Lu, K. P. (2002) *Mol Cell Biol* **22**(15), 5281-5295
50.   McCright, B., Rivers, A. M., Audlin, S., and Virshup, D. M. (1996) *J Biol Chem* **271**(36), 22081-22089
51.   Campaner, S., Kaldis, P., Izraeli, S., and Kirsch, I. R. (2005) *Mol Cell Biol* **25**(15), 6660-6672
52.   Treier M, S. L., Bohmann D. (1994) *Cell* **78**(5), 787-798.
53.   Wadzinski, B. E., Eisfelder, B. J., Peruski, L. F., Jr., Mumby, M. C., and Johnson, G. L. (1992) *J Biol Chem* **267**(24), 16883-16888
54.   Al-Murrani, S. W., Woodgett, J. R., and Damuni, Z. (1999) *Biochem J* **341 (Pt 2)**, 293-298
55.   Boudreau, R. T., and Hoskin, D. W. (2005) *Int Immunopharmacol* **5**(10), 1507-1518
56.   Qian, K. C., Wang, L., Hickey, E. R., Studts, J., Barringer, K., Peng, C., Kronkaitis, A., Li, J., White, A., Mische, S., and Farmer, B. (2005) *J Biol Chem* **280**(7), 6130-6137
57.   Richardson PG, M. C. (2005) *Future Oncol* **1**(2), 161-171
58.   Qian, K. C., Studts, J., Wang, L., Barringer, K., Kronkaitis, A., Peng, C., Baptiste, A., LaFrance, R., Mische, S., and Farmer, B. (2005) *Acta Crystallograph Sect F Struct Biol Cryst Commun* **61**(Pt 1), 96-99
59.   Li, X., and Virshup, D. M. (2002) *Eur J Biochem* **269**(2), 546-552
60.   Kim, K. T., Baird, K., Ahn, J. Y., Meltzer, P., Lilly, M., Levis, M., and Small, D. (2005) *Blood* **105**(4), 1759-1767
61.   Van Hoof, C., and Goris, J. (2004) *Cancer Cell* **5**(2), 105-106
62.   Shirogane, T., Fukada, T., Muller, J. M., Shima, D. T., Hibi, M., and Hirano, T. (1999) *Immunity* **11**(6), 709-719

63. Verbeek, S., van Lohuizen, M., van der Valk, M., Domen, J., Kraal, G., and Berns, A. (1991) *Mol Cell Biol* **11**(2), 1176-1179
64. Sears, R. C. (2004) *Cell Cycle* **3**(9), 1133-1137
65. Van Kanegan, M. J., Adams, D. G., Wadzinski, B. E., and Strack, S. (2005) *J Biol Chem* **280**(43), 36029-36036
66. Ugi, S., Imamura, T., Maegawa, H., Egawa, K., Yoshizaki, T., Shi, K., Obata, T., Ebina, Y., Kashiwagi, A., and Olefsky, J. M. (2004) *Mol Cell Biol* **24**(19), 8778-8789
67. Palaty, C. K., Kalmar, G., Tai, G., Oh, S., Amankawa, L., Affolter, M., Aebersold, R., and Pelech, S. L. (1997) *J Biol Chem* **272**(16), 10514-10521
68. Jacobs, M. D., Black, J., Futer, O., Swenson, L., Hare, B., Fleming, M., and Saxena, K. (2005) *J Biol Chem* **280**(14), 13728-13734
69. Bullock, A. N., Debreczeni, J., Amos, A. L., Knapp, S., and Turk, B. E. (2005) *J Biol Chem* **280**(50), 41675-41682
70. Xu YX, H. Y., Zhou XZ, Lu KP, Manley JL. . (2003) *Genes Dev.* **17**(22), 2765-2776.

FOOTNOTES

The abbreviations used are: BSA, bovine serum albumin; CHX, cycloheximide; DMEM, Dulbecco's modified Eagles's medium; DTT, dithiothreitol; FBS, fetal bovine serum; HEK 293T, human embryonic kidney 293T; OA, okadaic acid; PBS, phosphate buffered saline; PP2A, protein phosphatase 2A; SDS-sodium dodecyl sulfate polyacrylamide gel electrophoresis; shRNA, small hairpin RNA.

FIGURE LEGENDS

FIGURE 1. Pim-1 protein levels are negatively regulated by PP2Ac.A, Increased cellular PP2A activity decreases Pim-1 and Pim-2 protein levels. HEK-293T cells were contransfected with 0.5 µg pcDNA3/Pim-1(murine) (or Pim-2, RUNX1) and increasing amounts of pD30-PP2A-HA-C, 0.5 µg, 1 µg to 2 µg, as indicated. Control empty pcDNA3 vector was added to ensure that the total amount of plasmid DNA per transfection was the same. Whole-cell lysates were collected at 36 h post-transfection. Western blotting was carried out using anti-HA (for PP2Ac), anti-Pim-1, anti-Pim2, and anti-His to detect RUNX1 as a control. B, Inhibition of PP2A activity by Okadaic acid (OA) enhances the Pim-1 protein levels. HEK-293T cells were transfected with 0.1 µg pcDNA3/Pim-1. At 4 h before harvesting, transfected cells were either treated with ethanol (-), or increasing amounts of OA, 25 nM, 50 nM to 100 nM, as indicated. Whole-cell lysates were collected at 36 h post-transfection.

FIGURE 2. PP2A dephosphorylates Pim-1 both *in vivo* and *in vitro*, and decreases Pim-1 kinase activity. A, *In vivo* dephosphorylation assay. HEK 293T cells were cotransfected with 2 µg pcDNA3/Flag-Pim-1, and either pD30-PP2A-HA-C or empty vector, as indicated. After 36 h transfection, the Flag-Pim-1 proteins were labeled by incubating the cells in media containing [^{32}P] orthophosphate for 4 h followed by the immunoprecipitation of Flag-Pim-1 proteins. The immunoprecipitates were analyzed by autoradiography. B, *In vitro* dephosphorylation assay. HEK 293T cells were transfected with 2 µg pcDNA3/Flag-Pim-1. The [^{32}P] orthophosphate labeled Flag-tagged immunoprecipitates were treated

with or without 1 unit recombinant PP2A (A/C dimmer) *in vitro* phosphatase reactions, and analyzed by autoradiography. C, *In vitro* kinase assay. HEK 293T cells were transfected with 2 µg pcDNA3/Flag-Pim-1 and the indicated amount of PP2Ac. The Flag-tagged Pim-1 immunoprecipitates were used in a kinase assay with histone H1 as a substrate. A no-substrate reaction was used as a negative control (data not shown).

FIGURE 3. Pim-1 specifically associates with B56β *in vivo*. A, PP2A holoenzyme formation is required to negatively regulate Pim-1 protein levels. HEK-293 cells were cotransfected with 0.5 µg pcDNA3/Pim-1, with or without 0.5 µg pCEP-SV40 small T antigen, plus increasing amounts from 1 µg to 2 µg of pD30-PP2A-HA-C, as indicated. Whole-cell lysates were collected at 36 h post-transfection. B, Pim-1 coimmunoprecipitates with the B56β subunit. Pim-1 was contransfected with 0.5 µg HA tagged B56α, -β, -γ, -δ, -ε subunits into 293T cells. Whole-cell lysates were collected at 48 h posttransfection and immunoprecipitated with anti-HA beads. 5% of the lysates were saved for input detection and 50% of the immunoprecipitation samples were analyzed by Western blotting with anti-HA for B56 subunits, and anti-Flag for Pim-1. C, B56β coimmunoprecipitates with Pim-1. The experiment was carried out similarly to Fig.3B except that the cell lysates were immunoprecipitated with anti-Flag beads and western blotted with the appropriate antibodies as identified.

FIGURE 4. Knockdown of B56β increases Pim-1 protein stability and decreases Pim-1 uniquitinylation. A, Vector-expressed shRNA B56β specifically targeted to B56β, but not other B56 subunits. HEK-293T cells were contransfected with 0.5µg of either pCEP4HA- B56α, -β, -γ, -δ, or ε, as indicated, and 1.5 µg pSUPER-shRNA expression vector (empty vector or targeted to B56β). Cells were maintained in DMEM supplemented with 2% FBS for 72 h. Lysates were prepared and normalized. Immunoblots were probed for indicated B56 subunits with anti-HA antibody. B, Knockdown of B56β mRNA results in increased Pim-1 expression. HEK-293 cells were cotransfected with 0.2 µg pcDNA3/Pim-1, and increasing amounts from 0.5 µg to 2 µg of pSUPER-shRNA expression vector [empty (−) or targeted to PP2A B56β]. Cells were maintained for 72 h and lysates prepared and normalized. C, Stability of Pim-1 protein is increased upon B56β knockdown. 100-mm dishes of HEK-293 cells were cotransfected with 1 µg pcDNA3/Pim-1, and 4 µg shRNA-empty or shRNA-B56β for 24 h. Each transfection mixture was split into six 60-mm dishes with 10% FBS for 24 h and then starved in 0.2% FBS for 48 h. Cells were treated with 100 µg/ml cycloheximide (CHX), and cell lysates were prepared at the indicated time points after treatment. Pim-1 protein levels were quantified relative to GAPDH levels and graphed as percent Pim-1 protein remaining after cycloheximide treatment. Protein half-life was calculated using the Excel (Microsoft) graphing function. D, Ubiquitinylated Pim-1 protein is decreased by B56β knockdown. HEK-293T cells were contransfected with CMV-HA-ubiquitin, pcDNA3/Flag-Pim-1, and shRNA-empty or shRNA-B56β for 48 h. Cells were then maintained in DMEM containing 1% FBS with 1 µM Bortezomib for 6 h. Cells were harvested and lysates were divided for immunoprecipitaion with anti-Flag beads or for input detection. 5% of the lysates sample was used for input detection and 50% of the immunoprecipitation samples were analyzed by Western blotting with anti-ubiquitin antibody and anti-Pim-1.

FIGURE 5. The Pin1 Isomerase Associates with Pim-1 and B56β Facilitates Pim-1 Degradation. A, HEK-293T cells were contransfected with 1 µg CMV-HA-Pin1, and (or) 1 µg pcDNA3/Flag-Pim-1, 1 µg pCEP4HA-B56β, as indicated. Anti-Flag immunoprecipitations were carried out on cleared lysates. 5% percent of the extract was used to measure transfected protein levels while 50% of the immunoprecipitates were analyzed by Western blotting with anti-HA for the B56β, Pin1, and anti-Pim-1. B, Knockdown of Pin1 results in increased Pim-1 expression. *(Upper panel)*HEK-293T cells were contransfected with 0.5 µg HA/Pin1, and increasing amounts from 1 µg to 2 µg of shRNA-Pin1 (Sigma Mission shRNA, Cat No. TRCN0000001034). Cells were maintained in DMEM supplemented with 2% FBS for 72 h. Lysates were prepared and normalized for Western blotting. *(Bottom panel)* Cells were
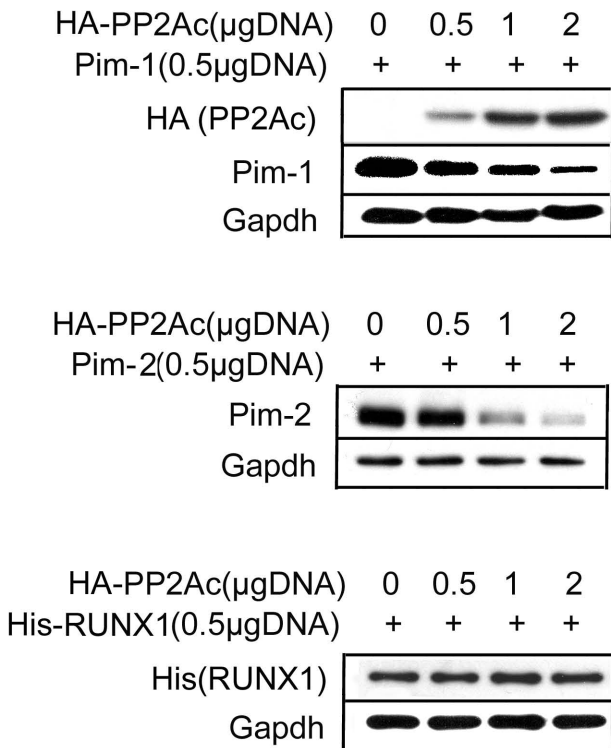
contransfected with 0.2 μg pcDNA3/Pim-1, and increasing amounts from 1 μg to 2 μg of shRNA-Pin1. Cells were maintained in DMEM supplemented with 2% FBS for 72 h.

FIGURE 6. Identification of Pim-1 domain responsible for banding to B56β and the binding properties of B56β deletion mutants to Pim-1. A, Structural domains of Pim-1 and Pim-1deletion mutants use in these experiments are represented as black bars. B, The B56β binds the hinge region of Pim-1. The HA-B56β (full length) was contransfected into HEK-293T cells with mock or the indicated Flag-tagged Pim-1 deletion mutants. The Flag-tagged Pim-1 proteins were immunoprecipitated and immunoblotted with anti-HA antibody to detect B56β or anti-Flag to measure Pim-1. C, Structural domains of B56β and B56β deletion mutants used in these experiments are represented as black bars. D, Identify the binding domain in B56β responsible for associating with Pim-1. The Flag-Pim-1(full length) was contransfected into HEK-293T cells with indicated HA-tagged B56β deletion mutants. The Flag-Pim-1 proteins were immunoprecipitated and the SDS-PAGE immunoblotted with anti-HA antibody to recognize B56β or anti-Flag to measure Pim-1. E. ASBD (206-389, A subunit binding domain 1 and 2) in B56β can bind to the Pim-1. F. Pim-1 inhibits B56β binding to A subunit. The Flag-A subunit, HA-B56β with or without HA-Pim-1 were cotransfected into HEK-293T cells. The Flag-tagged A subunit was immunopricipitated and the SDS-PAGE immunoblotted with anti-HA antibody to recognize B56β and Pim-1 or anti-Flag to measure A subunit.

FIGURE 7. Endogenous Pim-1 Protein levels are modulated by PP2Ac and B56β. A, Increased cellular PP2A activity decreases endogenous Pim-1 protein levels. BaF3 cells were transfected with increasing amounts from 0.5 μg, 1μg to 2 μg of pD30-PP2A-HA-C, and control empty pcDNA3 vector was added to ensure that the total amount of plasmid DNA per transfection was the same. Whole-cell lysates were collected at 36 h post-transfection and western blotted for Pim-1 levels. B, Inhibition of PP2A activity by Okadaic acid (OA) enhances endogenous Pim-1 protein levels. BaF3 cells were either treated with ethanol (-), or increasing amounts of OA ranging from 25 nM, 50 nM to 100 nM, as indicated, for 4 h. C, Knockdown of B56β results in increased endogenous Pim-1 expression. BaF3 cells were transfected with increasing amounts from 0.5 μg to 2 μg of pSUPER-shRNA expression vector [empty control or targeted to B56β]. Cell extracts were analyzed by western blotting. D, The effect of knockdown of B56β on BaF3 cell growth was analyzed by a modified MTT assay (MTS). BaF3 cells were transfected with either 2 μg of shRNA-empty or shRNA-B56β and allowed to grow for 48 h. 1,250 cells were then seeded in 96-wells in triplicate. After 48 hours, the cell viability was determined by MTS assay. Data shown is the means of three independent experiments; Error bars shown are the standard error with a student T-test indicating a significant difference $P < 0.05$(*), comparing experimental with the control group.
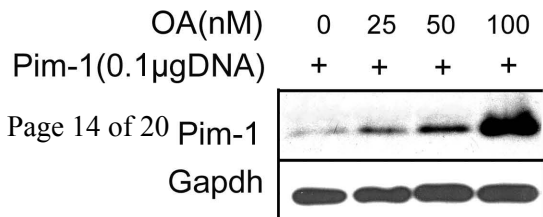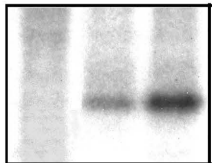
FIGURE 1

A

| HA-PP2Ac(µgDNA) | 0 | 0.5 | 1 | 2 |
| Pim-1(0.5µgDNA) | + | + | + | + |

HA (PP2Ac)

Pim-1

Gapdh

| HA-PP2Ac(µgDNA) | 0 | 0.5 | 1 | 2 |
| Pim-2(0.5µgDNA) | + | + | + | + |

Pim-2

Gapdh

| HA-PP2Ac(µgDNA) | 0 | 0.5 | 1 | 2 |
| His-RUNX1(0.5µgDNA) | + | + | + | + |

His(RUNX1)

Gapdh

B

| OA(nM) | 0 | 25 | 50 | 100 |
| Pim-1(0.1µgDNA) | + | + | + | + |

Page 14 of 20 Pim-1

Gapdh

FIGURE 2

A

In vivo dephosphorylation

| PP2Ac(4µgDNA) | - | + | - |
| Flag-Pim-1(2µgDNA) | - | + | + |

phos-Pim-1 $\longrightarrow$

B

In vitro dephosphorylation

| recombinant PP2A(1 unit) | - | + |
| Flag-Pim-1(2µgDNA) | + | + |

phos-Pim-1 $\longrightarrow$
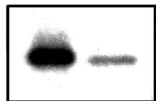
C

kinase assay

| PP2Ac(µgDNA) | 0 | 2 | 4 |
| Flag-Pim-1(2µgDNA) | + | + | + |

phos-Histone H1

FIGURE 3

A

Pim-1(0.5µgDNA)   +   +   +   +   +   +
SV40 ST(0.5µgDNA)   -   -   -   +   +   +
PP2Ac(µgDNA)   0   1   2   0   1   2

Pim-1

Gapdh

B

HA tagged   B56 α   B56 β   B56 γ   B56 δ   B56 ε
Flag-Pim-1   +   +   +   +   +

IP:HA(B56)
IB:Flag(Pim-1)

Heavy chain
←Pim-1
Light chain

IP:HA(B56)
IB:HA(B56)

Lysate:Pim-1

C

HA tagged   B56α   B56β   B56γ   B56δ   B56ε
Flag-Pim-1   +   −   +   −   +   −   +   −   +   −

IP:Flag(Pim-1)
IB:HA(B56)

IP:Flag(Pim-1)
IB:Flag(Pim-1)→

Lysate:
IB:HA(B56)

FIGURE 4

A

HA tagged →

IB: HA

	shRNA empty	shRNA B56β
B56α		
B56β		
B56γ		
B56δ		
B56ε		

B

	shRNA empty				shRNA B56β			
shRNA (µgDNA)	0	0.5	1	2	0	0.5	1	2
Pim-1(0.2µgDNA)	+	+	+	+	+	+	+	+

Pim-1

Gapdh

C

Cycloheximide(100µg/ml)

minutes 0 15 30 60 120 180

Pim-1
Gapdh
shRNA empty

Pim-1
Gapdh
shRNA B56β

Pim-1 half-life

% Pim-1 remaining vs Time(hrs)

● shRNA B56 β .t(1/2)=2.8h
▲ shRNA empty .t(1/2)=0.7h

D

	shRNA empty	shRNA B56β	
Flag-Pim-1	+	+	–
IP:Flag(Pim-1) IB:Ubiquitin			ubiquitinylated Pim-1
IP:Flag,IB:Flag			
Lysate:Pim-1			

FIGURE 5

FIGURE 6

FIGURE 7

A

PP2Ac(μgDNA) 0 0.5 1 2

Pim-1

← 44kD

← 33kD

Gapdh

B

OA(nM) 0 25 50 100
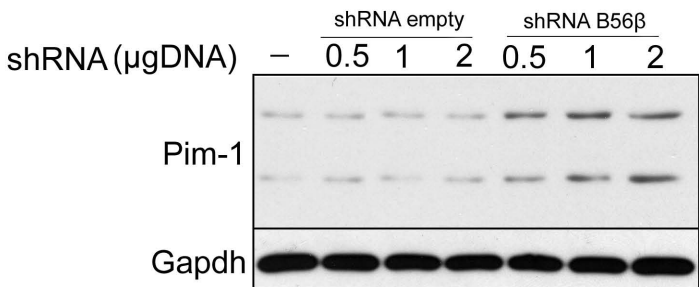
Pim-1

Gapdh

C

shRNA (μgDNA) − shRNA empty 0.5 1 2 shRNA B56β 0.5 1 2

Pim-1

Gapdh

D

□ shRNA empty ■ shRNA B56β

% viable cells

*